Citrate driven transamination for flavor production in *Lactococcus lactis*

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Citrate driven transamination for flavor production in *Lactococcus lactis*

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Chapter 1

Introduction

1

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1. The cheese bacterium Lactococcus lactis

Lactococcus lactis (formerly *Streptococcus lactis*) is a non-pathogenic Gram-positive bacterium that belongs to the Lactic Acid Bacteria (LAB) found in the phylum *Firmicutes*. Cells of lactococci are typically spherical or ovoid with a size of 1.2-1.5 µm that occur in pairs and short chains (10, 28). The natural habitat of *L. lactis* is related to plant or animal surfaces and the animal gastrointestinal tract. *L. lactis* is easily inoculated into milk, and therefore, domesticated species are used by the dairy industry as starters in cheese and other fermented food products. The main advantage of *Lactococcus* sp. is production of lactic acid by homofermentative degradation of sugars and production of aroma compounds from amino acids metabolism. Different strains of lactococci are involved in the manufacture of cheeses such as cheddar, colby, cottage cheese, cream cheese, camembert, roquefort and brie, as well as other dairy products like butter, buttermilk, sour cream, and kefir. Another application is fermentation of vegetables such as cucumber pickles and sauerkraut. The bacterium is used in single strain starter cultures or in mixed strain cultures with other LAB such as *Lactobacillus* and *Streptococcus* sp. (51, 75, 81, 83, 99).

The taxonomic structure of *L. lactis* is unusual (Table 1). Based on the sequence of the 16S rRNA gene, the species are divided into two subspecies: *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* (94). Historically, species have been classified in three industrially significant phenotypes: *L. lactis* subsp. *lactis* (lactis phenotype), *L. lactis* subsp. *cremoris* (cremoris phenotype), and *L. lactis* subsp. *lactis* biovar *diacetylactis* (diacetylactis phenotype). The lactis

Table 1. Phenotypes of *Lactococcus* species.

Organism[a]	Phenotype[b]	Growth[c]		Metabolism[c]			
		at 40 °C	4% NaCl	citrate	arginine	maltose	lactose galactose
L. lactis subsp. *lactis*	*lactis*	+	+	-	+	+	+
	cremoris	-	-	-	-	-	+
L. lactis subsp. *lactis* biovar *diacetylactis*	*diacetylactis*	+	+	+	+	+	+
L. lactis subsp. *cremoris*	*lactis*	+	+	-	+	+	+
	cremoris	-	-	-	-	-	+

[a] three industrial (historical) phenotypes (lactis, diacetylactis, cremoris);
[b] according to Kelly & Ward (2002);
[c] according to Schleifer *et al*. (1985) and Rademaker *et al*. (2007).

phenotype produces ammonia from arginine and is tolerant to high temperature (40 °C) and high salt concentration (4 % of NaCl). The cremoris phenotype is characterized by the inability to produce ammonia from arginine and by low tolerance to temperatures and NaCl concentrations. The diacetylactis phenotype is characterized by the ability to ferment citrate and to produce the flavor compound diacetyl (78). The genotypic and phenotypic results do not completely correspond; therefore, strains may have a lactis genotype with a cremoris phenotype and vice versa (90, 92).

L. lactis subsp. *lactis* with the lactis phenotype exists in a wide variety of environments. Some of these strains used as starters adapted to the dairy environment by acquisition of plasmids and other mobile genetic elements. The cremoris phenotype of the same subspecies occurs rarely. *L. lactis* subsp. *lactis* biovar *diacetylactis* is commonly used in milk fermentations due to the ability to metabolize citrate and is hardly found in other environments. The genes encoding the citrate metabolic pathway are located on the chromosome, in contrast to the gene encoding the citrate uptake system that is encoded on plasmids. The widely studied and sequenced strain IL1403 belongs to this group as its parent strain CNRZ157 is able to metabolize citrate due to the presence of a plasmid with the citrate uptake system. The *L. lactis* subsp. *cremoris* lactis phenotype is often associated with milk and plant environment, but is present in low numbers. Strain MG1363, which has been used for most of the biochemical and genetic research in *L. lactis*, belongs to this group. The cremoris phenotype of this subspecies is totally adapted to dairy and cannot survive in other environments. Strains from this group are widely distributed in starter cultures, mostly to produce cheddar cheese (47, 72).

2. Citrate fermentation

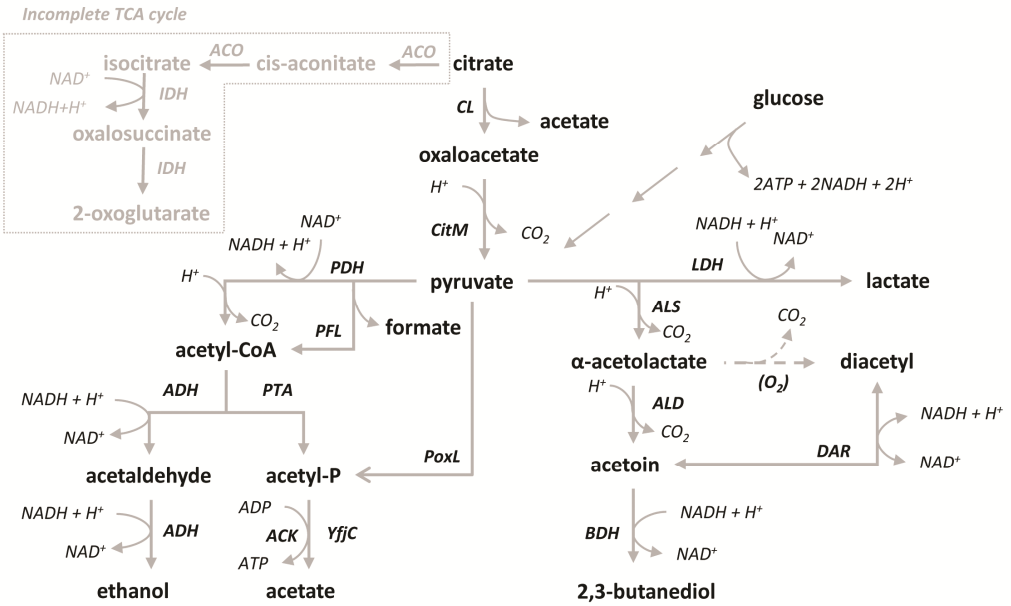
L. lactis subsp. *lactis* biovar *diacetylactis* is able to ferment citrate that is present in many of the substrates used for food fermentation such as milk, fruit, and vegetables. Citrate is also added as a preservative to foods. In milk, citrate constitutes almost 90 % of all organic acids and is present at concentrations ranging from 0.9 to 2.3 g/L (4-10 mM). During food fermentations, citrate is cometabolized with carbohydrates, f.i. with lactose that is present in milk at concentrations up to 5 g/L (14 mM) (74). Under normal conditions where sugars are not limiting and oxygen is confined, one molecule of glucose derived from lactose, is fermented to two molecules of L-lactic acid (homofermentative pathway). Conversion of carbohydrates to L-lactic acid in *L. lactis* follows the glycolytic Embden-Meyerhof-Parnas pathway (69). The produced lactate provides protection against spoilage by non-acidophilic organisms during food fermentation (69, 83, 99). The co-fermentation of lactose and citrate by LAB in milk is important for cheese industry because it leads to production of C4-aroma compounds such as acetoin, diacetyl, and 2,3-butanediol (32, 39). Acetoin and diacetyl are responsible for the buttery flavor of dairy products such as butter,

margarine, ice-creams, and cottage cheese, 2,3-butanediol gives a fruity-like odor (81).

The genome of *L. lactis* subsp. *lactis* biovar *diacetylactis* IL1403 encodes enzymes responsible for citrate metabolism yielding end products such as C4-aroma compounds, ethanol, acetate, and L-lactate (Fig. 1) (10). A short part of the Krebs cycle (tricarboxylic acid cycle) from citrate to α-ketoglutarate is annotated in the genome; however, it has never been demonstrated to be active in *L. lactis* (91). The main pathway of citrate metabolism consists of two enzymes: citrate lyase (CL) and soluble oxaloacetate decarboxylase (CitM) (3, 39, 57, 60). Genes involved in the biosynthesis of these two enzymes are located in the same *cit* operon (*citM-citCDEFXG*) and are induced by natural acidification of the medium (62). Citrate lyase subunits are encoded by the *citD*, *citE*, and *citF* genes, the accessory subunits required for the synthesis of an active complex are encoded by *citC*, *citX*, and *citG*, and oxaloacetate decarboxylase is encoded by the *citM* gene (*mae* in *L. lactis* IL1403) (10). Following uptake into the cell, citrate is converted into oxaloacetate and acetate by the activity of citrate lyase. Then, oxaloacetate is decarboxylated by oxaloacetate decarboxylase yielding pyruvate and carbon dioxide.

Cometabolism of citrate and glucose results in higher concentrations of internal pyruvate (Fig. 1) (3, 60, 73), whose metabolism yields various end products (Fig. 1). Production of C4-compounds

Figure 1. Possible routes of citrate metabolism in *Lactococcus lactis* IL1403.



ACK, acetate kinase; ACO, aconitase; ADH, acetaldehyde dehydrogenase; ALD, α-acetolactate decarboxylase; ALS, α-acetolactate synthase; BDH, 2,3-butanediol dehydrogenase; CitM, soluble oxaloacetate decarboxylase; CL, citrate lyase; DAR, diacetyl-acetoin reductase; IDH, isocitrate dehydrogenase; LDH, L-lactate dehydrogenase; PDH, pyruvate dehydrogenase complex; PFL, pyruvate-formate lyase; PoxL, pyruvate oxidase; PTA, phosphate acetyltransferase; TCA cycle, tricarboxylic acid cycle; YfjC, acylphosphate phosphohydrolase. Dashed lines indicate non-enzymatic reactions. Pathways are based on annotations of the genome sequence published by Bolotin *et al*. (2001).

is initiated by α-acetolactate synthase (ALS) that converts two molecules of pyruvate to one molecule of α-acetolactate while releasing carbon dioxide. The enzyme is encoded by the *als* gene located next to the *cit* operon and has a K_m of 50 mM for pyruvate (26, 84). A small part of the chemically unstable α-acetolactate results in the formation of diacetyl in a non-enzymatic oxidative decarboxylation reaction (38, 73), but the majority is decarboxylated to acetoin by α-acetolactate decarboxylase (ALD). The latter enzyme is encoded by the *aldB* gene located in the *leu-ilv-ald* operon that is responsible for the biosynthesis of branched-chain amino acids. Inactivation of *aldB* by a single mutation improved diacetyl formation due to enhanced spontaneous decarboxylation of accumulated α-acetolactate (35). Acetoin is reduced at the expense of NADH to 2,3-butanediol by activity of 2,3-butanediol dehydrogenase (BDH) or oxidated to diacetyl by activity of acetoin reductase (DAR) (32).

Next to the C4-compounds pathway, pyruvate is metabolized to L-lactate in the homofermentative pathway or to ethanol and/or acetate in the heterofermentative pathway (Fig. 1) (39). Production of L-lactate is catalyzed by L-lactate dehydrogenase (LDH) at the expense of NADH. The genome of *L. lactis* IL1403 provides three genes of L-lactate dehydrogenase, of which the protein encoded by *ldh* gene is known as the major L-lactate dehydrogenase. The product of the *ldhB* gene is active when *ldh* is mutated, and the *ldhX* gene encodes a protein of unknown function (10, 34). The heterofermentative route requires production of acetyl-CoA by the pyruvate dehydrogenase complex (PDH) or by pyruvate-formate lyase (PFL). The pyruvate dehydrogenase complex has a K_m of 1 mM for pyruvate and is very sensitive to NADH inhibition. It consists of four subunits encoded by the *pdhA*, *pdhB*, *pdhC*, and *pdhD* genes (10, 84). The pyruvate-formate lyase encoded by the *pfl* gene is strongly inhibited by oxygen (68). Acetyl-CoA is converted to ethanol through acetaldehyde at the expense of NADH by alcohol dehydrogenase (ADH) (encoded by *aldA*) or to acetate through acetyl phosphate by phosphate acetyltransferase (PTA) (encoded by *pta*) and acetate kinase (ACK) (encoded by *ackA1* and *ackA2*). The pathway from pyruvate to acetate via acetate kinase produces metabolic energy in the form of ATP. A shift from homolactic fermentation (L-lactate) to mixed-acid fermentation (ethanol and acetate) has been observed under certain growth conditions such as limited carbon and energy source (12), change in pH (88), limited oxygen (8, 21), change in the $NADH/NAD^+$ ratio (33, 85), or the presence of cofactors (86).

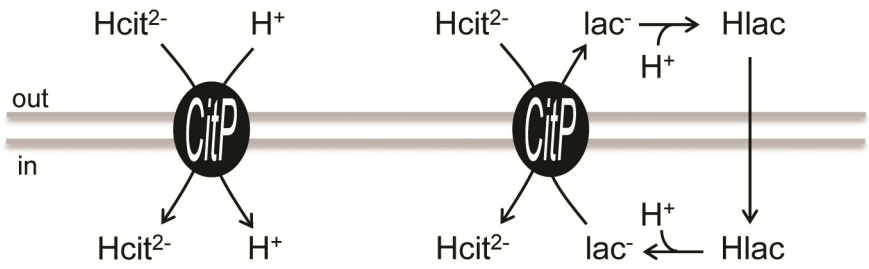
In *L. lactis* IL1403, another route from pyruvate to acetate is possible, which does not generate ATP. This pathway consists of two novel enzymes: pyruvate oxidase (PoxL) (encoded by *poxL*) and acylphosphate phosphohydrolase (YfjC) (encoded by *yfjC*) (10). Pyruvate oxidase uses oxygen to convert pyruvate into acetyl phosphate, carbon dioxide, and hydrogen peroxide. The enzyme plays a main role in the aerobic formation of acetate in *Lactobacillus plantarum* and the expression level of pyruvate oxidase gene is enhanced by oxygen or hydrogen peroxide and reduced by glucose (58). The pathway is related to NADH oxidases (NOX enzymes) that are important in controlling the $NADH/NAD^+$ balance in the cell (45).

3. The citrate transporter CitP

Citrate metabolizing genes in strains of *L. lactis* subsp. *lactis* biovar *diacetylactis* are located on the chromosome. Citrate uptake is catalyzed by the citrate transporter CitP and the encoding *citP* gene is always encoded on plasmids (47, 72). Loss of the plasmid results in the loss of citrate uptake and, consequently, diacetyl production (11). Restoration of this activity is experimentally possible, as was demonstrated by transformation of the plasmid free strain *L. lactis* IL1403 (17) with plasmid pFL3 containing the lactococcal *citP* gene under control of the *Streptococcus pneumoniae polA* promoter (59).

Citrate metabolism in LAB is a metabolic energy generating pathway. The pathway generates an electrochemical gradient of protons (proton motive force, pmf) across the cell membrane (3, 64, 65) by a secondary mechanism in which membrane potential and pH gradient are generated in separate steps (55, 56). The transporter CitP catalyzes uptake of divalent citrate in exchange for monovalent lactate which results in a membrane potential of physiological polarity (Δψ, inside negative) (Fig. 2). The pH gradient (ΔpH, inside alkaline) is the result of proton consumption in the decarboxylation reactions taking place in the cytoplasm. The pathway functions as an indirect proton pump (55, 56).

Figure 2. Two modes of citrate transport by CitP operating and the shuttle mechanism in the presence of permeative L-lactate.



Left, CitP operating in the H^+ symport mode. Right, CitP operating in the exchange mode. L-Lactate added at the outside of the cells allows CitP to operate in the fast $Hcit^{2-}/lac^-$ exchange mode by reentering the cell in the permeative protonated state (Hlac).

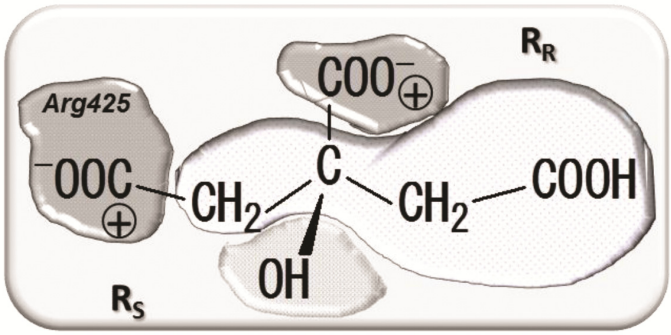
The citrate transporter CitP is a secondary transporter that belongs to the 2-hydroxycarboxylate transporter (2-HCT) family (Transporter Classification (TC) 2.A.24) (77). Secondary transporters represent the largest functional category of transporters, comprising over 100 families. To drive transport, these proteins use the free energy stored in electrochemical ion and/or solute gradient across the membrane. 2-HCT secondary transporters are found exclusively in bacteria and share specificity for substrates containing a 2-hydroxycarboxylate (2-HC) motif $HO\text{-}CR_2\text{-}COO^-$. All

characterized 2-HC transporters catalyze the uptake of either citrate or (S)-malate coupled to either a proton or a sodium ion gradient or both to drive uptake, f.i. the Na^+-citrate symporter CitS of *Klebsiella pneumonia*, the H^+-malate/citrate symporter CimH of *Bacillus subtilis*, and the H^+-malate symporter MalP of *Streptococcus bovis* (87). In addition, some of the 2-HC transporters, i.e. the citrate transporter CitP and the malate transporter MleP of LAB, exchange external divalent citrate or (S)-malate for internal monovalent lactate, the breakdown product of citrolactic and malolactic fermentation, respectively (precursor/product exchange). These two secondary transporters differ from the other members because they catalyze efficient heterologous exchange of two structurally related substrates and they translocate net negative charge across the membrane that allows for the generation of metabolic energy for the cells by creating a membrane potential (2, 3, 4, 64, 65, 87). Therefore, the 2-HCT family of secondary transporters contains both metabolic energy-dissipating (CitS, CimH, and MalP) and -generating (CitP and MleP) members.

The citrate transporter CitP was extensively characterized by transport studies in membrane vesicles (2, 3, 64). Kinetic studies of CitP revealed two modes of transport, symport of divalent citrate with one proton and exchange of divalent citrate with monovalent L-lactate (Fig. 2) (2, 3, 57, 64). Since the former was much slower than the latter, it was concluded that CitP is a symporter that has been optimized to catalyze exchange under physiological conditions during citrate/carbohydrate cometabolism in LAB. Transport studies *in vitro* using right-side-out (RSO) membrane vesicles derived from *L. lactis* demonstrated that CitP has affinity for 2-hydroxycarboxylates of the form $HO\text{-}CR_2\text{-}COO^-$, in which the R group ranged from a hydrogen atom in glycolate to a phenyl group in mandalate and to acetyl groups in malate and citrate (2). The transporter was shown to discriminate between high affinity substrates that contain a second carboxylate group in one of the R substituents like citrate and malate, and low affinity substrates, monocarboxylates like lactate, suggesting an important role of the second carboxylate group in the interaction with the protein. Based on the experiments, a model of the binding site of CitP was proposed (Fig. 3), in which the carboxylate and hydroxyl group of the 2-hydroxycarboxylate motif present in all substrates interact with specific sites on the protein (2, 4). This would fix the orientation of the substrate in the binding pocket and define two separate sites in the binding pocket (R_S and R_R) (Fig. 3) for optional interactions with the R groups of the substrates, including the interaction with a second carboxylate in the R_S site that results in high affinity binding. In agreement, the (S)-enantiomers of chiral dicarboxylate substrates like malate were bound with high affinity and the (R)-enantiomers with low affinity, whereas both enantiomers of monocarboxylates like lactate were bound with low affinity (4).

Site directed mutagenesis of CitP of *Leuconostoc mesenteroides,* another LAB used in the dairy industry with a sequence almost identical to the lactococcal *citP*, identified the conserved Arg425 residue as the site specifically interacting with the second carboxylate present in citrate and (S)-

Figure 3. Schematic model of the substrate binding pocket of CitP.



The substrate depicted in the pocket is citrate. The interactions between the carboxylate groups and the hydroxyl group of the substrate and the protein were indicated as grey surfaces, the hydrophobic interaction site (R_R) by a white surface. The R_S and R_R sites bind the side chains of the (S)- and (R)-enatiomers of monosubstituted 2-hydroxycarboxylates (HO-CHR-COO^-), respectively. Residue Arg425 responsible for binding of a carboxylate in the R side chain, when present, was indicated in the R_S site. The figure was modified from Bandell & Lolkema (1999) and Bandell *et al.* (2000).

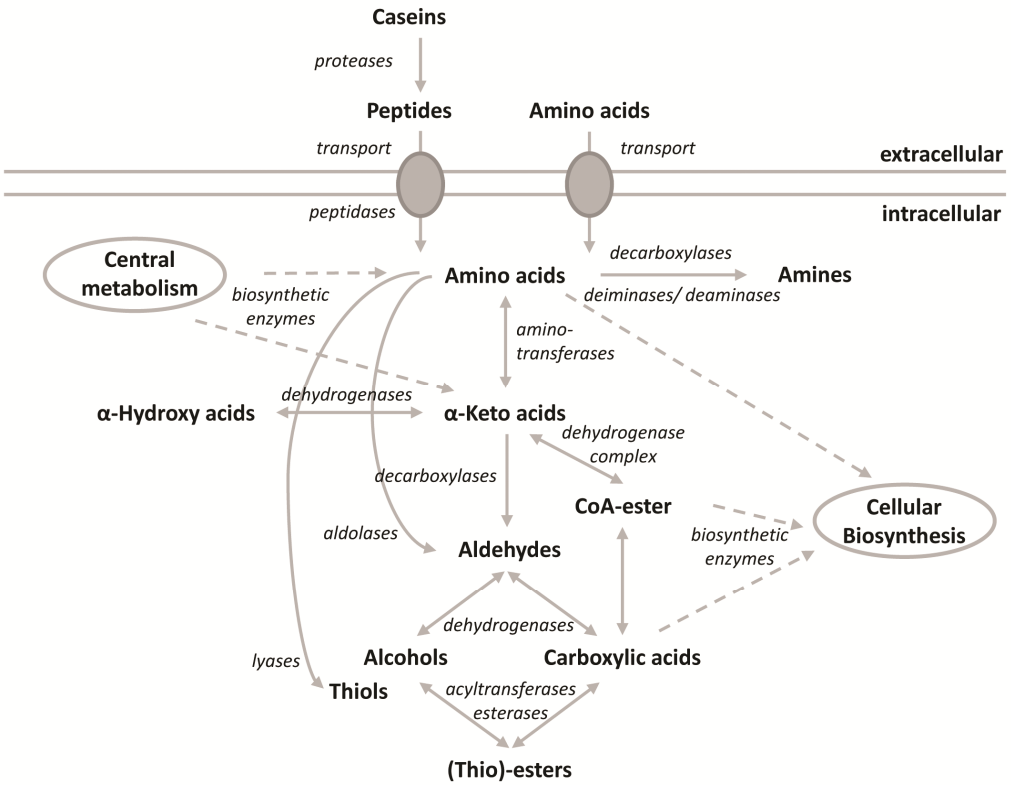
divalent substrates (5). Additionally, increasing binding affinity of monocarboxylates with increasing hydrophobicity of the R groups suggested a hydrophobic nature of the R_R and R_S sites. Evidence was put forward that at least part of the hydrophobic sites are located in the C-terminal 46 residues (4). While the carboxylate group of the 2-hydroxycarboxylate motif was essential for the interaction with CitP, the transport studies in RSO membranes showed that the hydroxyl group of the motif could be replaced to some extent by a keto group, like in oxaloacetate and pyruvate (2). The specificity of CitP for substrates carrying different charges forms the mechanistic basis for membrane potential generation (2, 4).

4. Amino acid metabolism in Lactic Acid Bacteria (LAB)

The ability of different *Lactococcus* sp. to synthesize amino acids is limited. The requirements for a nitrogen source necessary for growth appears to be strain dependent, but most *Lactococcus lactis* strains need isoleucine, leucine, valine, histidine, and methionine (18). Although milk is a protein-rich environment, the concentrations of free amino acids, especially those that are essential (Ile, Leu, and Met) are very low (43). To obtain all essential amino acids, *L. lactis* is able to degrade milk proteins from the casein family into small peptides and free amino acids that can be taken up from the environment by transporters (Fig. 4) (19). The process of casein utilization has previously been extensively studied and is initiated by an extracellular cell envelope-located protease PrtP. Two types of proteinases have been identified among lactococci on the basis of their specificity towards caseins, the P_I and P_{III} type. The P_{III} type hydrolyze more efficiently α-$_{s1}$ and κ-casein, but both types act preferentially on β-casein that is cleaved by the two enzymes in a different manner (44). Uptake of formed peptides is catalyzed by the oligopeptide transport

system (OPP system) and di/tri-peptide transporters (15, 24, 50, 52). Subsequently, internalized peptides are hydrolyzed yielding free amino acids by cytoplasmic peptidases (Pep enzymes) (19). Free amino acids in the medium may be transported into the cell by various uptake systems (30, 49).

Figure 4. Overview of metabolic pathways relevant for flavor compounds formation in dairy fermentations.



This figure was adapted from Smit *et al*. (2005).

Most of intracellular amino acids can be converted by aminotransferases to the corresponding α-keto acids. Transamination is the first step of flavor compound formation from branched-chain amino acids, aromatic amino acids, and methionine. α-Keto acids are central intermediates that can be decarboxylated into aldehydes or dehydrogenated into α-hydroxy acids or CoA-esters. The conversion of α-keto acids into α-hydroxy acids is catalyzed by NAD(H)-dependent 2-hydroxyacid dehydrogenases that may act as a sink for excessive redox equivalents (NADH). Aldehydes can be dehydrogenated or hydrogenated to their corresponding alcohols and carboxylic acids that are substrates to produce (thio)-esters. All described reactions are mostly enzymatic, but chemical conversion of some have also been described (30, 81, 83), f.i. chemical oxidation of phenylpyruvate (α-keto acid of phenylalanine) into benzaldehyde (70).

In addition to transamination, amino acids may be converted by lyases or aldolases to produce thiols or aldehydes, respectively (30, 83). For instance, threonine aldolase coverts threonine into acetaldehyde (16). Finally, amino acids can be deiminated/deaminated or decarboxylated yielding biogenic amines (30). The latter pathway has been studied extensively in relation to the presumed health risk of biogenic amines (13).

4.1 Transaminases

Aminotransferases are widely distributed in all living organisms and are involved in either biosynthesis or degradation of nearly all of the 20 proteogenic α-amino acids. They belong to the group of pyridoxal-5'-phosphate (PLP)-dependent enzymes that catalyze a transfer of amino groups from amino acids to α-keto acids. The PLP cofactor binds to the active center of aminotransferase by a Schiff base linkage that is formed between the aldehyde group of PLP and a specific lysine residue of the active center (67). The transfer of amino group follows the ping-pong Bi-Bi mechanism (20), in which the amino group from amino acid donor resides temporarily on the cofactor PLP to form pyridoxamine-5'-phosphate (PMP). PLP is further regenerated from PMP via the α-keto acid donor (see below) (48, 93).

$$E_L + \text{amino acid donor} \leftrightarrow E_M + \text{new keto acid}$$
$$E_M + \text{keto acid donor} \leftrightarrow E_L + \text{new amino acid}$$

E_L and E_M refer to the PLP and PMP forms of the enzyme, respectively.

Structure and sequence analysis of the PLP dependent enzymes has revealed seven different structural families ('fold types') (Table 2). Bacterial aminotransferases are found in six subfamilies that belong to the fold I or IV (79). Most of bacterial enzymes are classified in the fold type I aspartate aminotransferase family (AAT_like) that was named after the most extensively characterized PLP enzyme aspartate aminotransferase (AspAT). AspAT catalyzes the reversible transfer of an α-amino group between aspartate and glutamate and is widely distributed in archaea, bacteria, and eukaryotes (67). Most transaminases possess very broad substrate specificity, f.i. OAT_like transaminases from acetyl ornithine aminotransferase subfamily fold I catalyze the transamination of basic α-amino acids, ω-amino acids, and diamines (41). The common feature of these enzymes is their overlapping substrate specificity, which often leads to the non-existence of a phenotype after knocking out one of them. As an example, the final step in the synthesis of phenylalanine in *Escherichia coli* K-12 is a transamination reaction catalyzed by three different enzymes: aspartate transaminase AspC, aromatic aminotransferase TyrB, and branched-chain amino acids transaminase IlvE (9).

Table 2. Classification of the bacterial aminotransferases.

Fold type	Family	Family name[a]	Subfamily	Subfamily name[a]	Class[b]
I	Aspartate aminotransferase	AAT_I	Aspartate AT	AAT_like	I. Aspartate and aromatic AT
			Acetyl ornithine AT	OAT_like	III. Ornithine AT
			Phosphoserine AT	PSAT	IV. Phospho-serine AT
			Alanine-glyoxylate AT	AGAT	II. Glycine AT
II	Tryptophan synthase beta	Trp-synth-beta_II			
III	Alanine racemase	Ala_racemase			
IV	Pyridoxal 5'-phosphate dependent enzymes	PLPDE_IV	Branched-chain aminotransferase	BCAT_beta	
			D-Alanine aminotransferase	D-AAT_like	
V	Glycogen phophorylase				
VI	D-lysine 5,6-aminomutase				
VII	Lysine 2,3-aminomutase				

[a] in NCBI database;
[b] according to Jensen & Gu (1996) and Mehta & Christen (2000);
AT, aminotransferase.

4.2 Transaminases of Lactococcus lactis IL1403

Genome analysis of *L. lactis* subsp. *lactis* IL1403 has provided 13 genes encoding putative aminotransferases (Table 3) (10). Four enzymes of *L. lactis:* AraT (96), BcaT (98), AspC (27), and YtjE (63) were purified and characterized. AlaT (61), PSAT (36), GlmS (46) are highly similar in sequence to the purified enzymes studied in other bacteria and ArcT, HisC (61), SPT (80), NAcOATase (54), NifS (89), and NifZ were annotated purely based on homology. The best keto

Table 3. Characterization of putative transaminases in *Lactococcus lactis* IL1403.

Subfamily[a]	Gene	Enzyme	Cellular function	AA[b]	KA[c]	Reference	Homology (%)
AAT_like	*aspB*	**AspC** aspartate aminotransferase (EC 2.6.1.1) Asp + α-KG ↔ OA + Glu	Aspartate biosynthesis	Asp Glu	OA α-KG	AspC in *L. lactis* (27)	100
				Asp Glu Trp Tyr Phe Met Cys	α-KG	AspAT in *Bacillus stearothermophilus* (7)	53
	aspC	**AlaT** alanine aminotransferase (EC 2.6.1.2) Ala + α-KG ↔ Pyr + Glu	Alanine biosynthesis	Ala Glu Asp Gln	Pyr α-KG α-KB	AlaT in *Corynebacterium glutamicum* (61)	55
	araT	**AraT** aromatic amino acid aminotransferase (EC 2.6.1.57) Phe + α-KG ↔ α-KPhe + Glu	Aromatic amino acids degradation	Glu Leu Tyr Phe Trp Met	α-KG α-KLeu α-KTyr α-KPhe α-KTrp α-KMet	*L. lactis* (97)	97

	ytjE	**YtjE** aminotransferase (EC 4.4.1.1)	Cysteine and methionine metabolism	Met Cys Glu	α-KG	YtjE in *L. lactis* (63)	99
	arcT	**ArcT** aminotransferase	Arginine catabolism			AT in *Lactobacillus, Streptococcus, Enterococcus* sp.	51-55
	hisC	**HisC** histidinol-phosphate aminotransferase (EC 2.6.1.9)	Histidine, tyrosine, and phenylalanine biosynthesis	Glu Asp	α-KB α-KLeu	HisC in *Corynebacterium glutamicum* (61)	29
OAT_like	*argD*	**NAcOATase** N-acetylornithine aminotransferase (EC 2.1.1.11)	Lysine, arginine, proline, and ornithine biosynthesis	Glu	α-KG	NAcOATase (*argD*) in *E. coli* (54)	38
PSAT	*serC*	**PSAT** phosphoserine aminotransferase (EC 2.6.1.52)	Serine biosynthesis	Glu	α-KG	PSAT (*serC*) in *E. coli* (36)	49
PSAT	*yeiG*	**SPT** serine:pyruvate transaminase (EC 2.6.1.51)	Glycine, serine, and threonine metabolism	Ser Ala	Pyr	SPT in *L. lactis* (80)	99
						SPT from rat liver (71)	27

Ser + Pyr ↔ α-KSer + Ala

BCAT_beta	*bcaT*	**BcaT** branched-chain amino acid aminotransferase (EC 2.6.1.42)	Valine, leucine, isoleucine metabolism	Glu Ile Leu Val Met	α-KG α-KIle α-KLeu α-KVal α-KMet	BcaT or IlvE in *L. lactis* (98)	99
		Ile + α-KG ↔ α-KIle + Glu					
AAT_I family, class V	*nifS*	**NifS** PLP-dependent aminotransferase (EC 2.8.1.7)	Cysteine/thiamin metabolism	Cys Ala		Cystein desulfurase (*iscS*) in *E. coli*	38
				Glu	α-KG	Cysteine desulfurase (*nifS*) in *Bradyrhizobium japonicum* (89)	37
	nifZ	**NifZ** PLP-dependent aminotransferase (EC 2.8.1.7)				Cystein desulfurase in *Lactobacillus, Streptococcus, Enterococcus, Bacillus* sp.	45-58
GFAT-type (not PLP-dependent)	*glmS*	**GlmS** glucosamine-fructose-6-phosphate aminotransferase (EC 2.6.1.16)	Glutamine metabolism	Glu Gln		GlmS in *Streptococcus* sp. (46)	80

[a] in NCBI database;
[b] amino acid donor characterized experimentally;
[c] keto acid donor characterized experimentally;
GFAT-type, Glutamine amidotransferases class-II; OA, oxaloacetate; Pyr, pyruvate; α-KB, α-ketobutyrate; α-KG, α-ketoglutarate; α-KIle, 2-ketomethylvalerate; α-KLeu, α-ketoisocaproate; α-KMet, 4-methylthio-2-ketobutyrate; α-KPhe, β-phenylpyruvate; α-KSer, 3-hydroxypyruvate; α-KTrp, indole-3-pyruvate; α-KTyr, *p*-hydroxyphenylpyruvate; α-KVal, α-ketoisovalerate. Transaminases indicated by grey surface may be involved in aroma compounds development in IL1403.

donor for transamination in LAB is α-ketoglutarate. Therefore, most experimental studies in *L. lactis* were performed with this keto acid (75, 91, 97, 99). The two lactococcal aminotransferases AraT and BcaT were demonstrated as the major enzymes that convert aromatic amino acids, branched-chain amino acids, and methionine to the corresponding α-keto acids (97, 99). The latter are further metabolized to aroma compounds important in cheese (Fig. 4) (75). Both enzymes were isolated from *L. lactis* subsp. *cremoris* NCDO763 and are identical in amino acid sequence to those encoded in strain IL1403. The substrate specificities of the purified enzymes were studied. AraT efficiently catalyzed α-ketoglutarate transamination with leucine, tyrosine, phenylalanine, tryptophan, and methionine. Phenylalanine or leucine was transaminated with the keto acids α-ketoisocaproate, *p*-hydroxyphenylpyruvate, β-phenylpyruvate, indole-3-pyruvate, and 4-methylthio-2-ketobutyrate (Table 3). No activity was measured with isoleucine, valine, histidine, aspartate, alanine, cysteine, lysine, proline, α-ketomethylvalerate, α-ketoisovalerate, and oxaloacetate (97). BcaT was shown to catalyze the reaction between α-ketoglutarate and isoleucine, leucine, valine, or methionine, and between isoleucine and α-ketoisocaproate, α-ketoisovalerate, 4-methylthio-2-ketobutyrate. No activity was measured with alanine, cysteine, phenylalanine, β-phenylpyruvate, pyruvate, and oxaloacetate (98). Both transaminases play a major role in the regulation of the expression of the proteolytic system in *L. lactis*. The intracellular pool of branched-chain amino acids is regulated by these enzymes that are part of the CodY regulon. The pleiotropic transcriptional regulator CodY represses the expression of the proteolytic system as well as both AraT and BcaT (14).

Double inactivation of *araT* and *bcaT* genes in *L. lactis* completely inhibited α-ketoglutarate transamination with aromatic amino acids, branched-chain amino acids, and methionine with no effect on the degradation of aspartate indicating the presence of another transaminase specific for aspartate/oxaloacetate (75). One aspartate transaminase AspC (encoded by the *aspB* gene) was identified (10). AspC of strain IL1403 is identical in sequence to the functionally characterized enzyme of *L. lactis* subsp. *cremoris* LM0230. A crude cell extract of *E. coli* cells expressing the latter lactococcal gene showed α-ketoglutarate transamination activity only in the presence of aspartate out of 20 tested amino acids (27). In contrast, the AspC homolog (53 % sequence identity) purified from *Bacillus stearothermophilus* revealed additional substrate specificity towards tryptophan, tyrosine, phenylalanine, methionine, and cysteine without specificity towards valine, isoleucine, leucine, proline, glycine, serine, threonine, asparagine, glutamine, lysine, arginine, and histidine (7).

None of the three enzymes discussed above (AraT, BcaT, or AspC) was able to catalyze transamination of alanine (pyruvate). A single protein in IL1403 was annotated as pyruvate specific transaminase, the alanine aminotransferase AlaT (encoded by *aspC*) (10); however, the gene product has not been studied in *Lactococcus* sp.. The homologous protein from *Corynebacterium glutamicum* (55 % of sequence identity) showed high activity for alanine and glutamate (pyruvate and α-ketoglutarate) and low activity for aspartate and glutamine (61). The

yeiG gene in the genome of IL1403 was annotated as serine:pyruvate transaminase SPT. SPT was purified and characterized from eukaryotic cells and shares only 27-30 % sequence identity with the lactoccocal homologues (71). The SPT encoding gene is also found in strain *L. lactis* subsp. *lactis* KF147 (80).

Recently, the product of the *ytjE* gene that was annotated as an aminotransferase was isolated and functionally characterized. Rather than being a specific transaminase for methionine as suggested (10), YtjE showed C-S lyase activity with α, γ-elimination that results in the formation of flavor compounds such as MTL, DMTS, and DMDS in the presence of methionine (63).

The other six enzymes HisC, NAcOATase, PSAT, NisF, NifZ, and GlmS (encoded by *hisC*, *argD*, *serC*, *nifS*, *nifZ*, and *glmS* genes, respectively) are very specific for one particular substrate, and are involved in amino acid biosynthesis pathways that are not related to aroma compounds formation. HisC catalyzes the formation of L-histidinol phosphate from imidazole-acetol phosphate and glutamate in the histidine biosynthesis pathway (61). NAcOATase catalyzes formation of N-acetyl-L-glutamate 5-semialdehyde from N2-acetyl-L-ornithine and α-ketoglutarate in arginine biosynthesis (54). SerC catalyzes the formation of 3-phosphonooxypyruvate from O-phospho-L-serine and α-ketoglutarate in serine biosynthesis (36). NifS and NifZ are cysteine desulfurases that function in the conversion of L-cysteine to L-alanine and sulfane sulfur via the formation of a protein-bound cysteine persulfide intermediate on a conserved cysteine residue (89). GlmS catalyzes conversion of fructose-6-phosphate into glucosamine-6-phosphate using glutamine as a nitrogen source in sugar metabolism (46). The gene product of *arcT* is associated with the arginine deiminase pathway genes and probably plays a role in arginine catabolism (10).

4.3 Formation of flavor compounds by amino acids catabolism

The major aroma compounds produced during amino acid catabolic pathways are aldehydes, alcohols, carboxylic acids, and (thio)-esters (Fig. 4) derived from branched-chain amino acids (isoleucine, leucine, valine), aromatic amino acids (phenylalanine, tyrosine, tryptophan), and methionine. α-Keto acids and α-hydroxy acids are not major flavor compounds themselves, but they play role in flavor development in semi hard cheeses made with lactococci (83, 97). Additionally, α-hydroxy acids exhibit antifungal and antilisterial activities important during cheese production (25, 53, 97). The final flavor of cheeses depends on the respective concentrations of the different key aroma compounds (99). Examples of aroma compounds derived from amino acids metabolism, which are important in flavor development in gouda, cheddar, camembert, and swiss-type cheeses are listed in Table 4. Amino acid metabolic pathways resulting in these compounds are described below.

Table 4. Important aroma compounds derived from amino acid catabolic pathways.

Metabolism	Flavor	Description
Ile	2-methylbutanal	malty, chocolate
	2-methylbutanol	wine
Leu	3-methylbutanal	malty, powerful, cheese
	3-methylbutanol	fresh cheese, breathtaking, alcoholic
	3-methylbutyrate	rancid, sweat, cheese, putrid
Val	2-methylpropanal	banana, malty, chocolate-like
Phe	phenylacetaldehyde	rose-like odour, floral
	benzaldehyde	bitter almond oil, characteristic sweet cherry
	phenylacetate	flowery-like odour
	phenylethanol	rosy-like odour
Trp	indole-	mothball
	skatole	unclean, mothball, fecal
Tyr	*p*-cresol	unclean, medicinal, cowy, barny
Met	methional	cooked potato, meat like, sulphur
	methanethiol	“rotting” cabbage, cheese, vegetative, sulphur
	methylthiopropionate	baked potato
	dimethyl sulfide (DMS)	cabbage, sulfurous
	dimethyl disulfide (DMDS)	onion, sulfurous
	dimethyl trisulfide (DMTS)	cabbage, garlic, sulfurous
	dimethyl tetrasulfide (DMQS)	putrid, cabbage, sulfurous
Thr	acetaldehyde	yoghurt, green, nutty, pungent

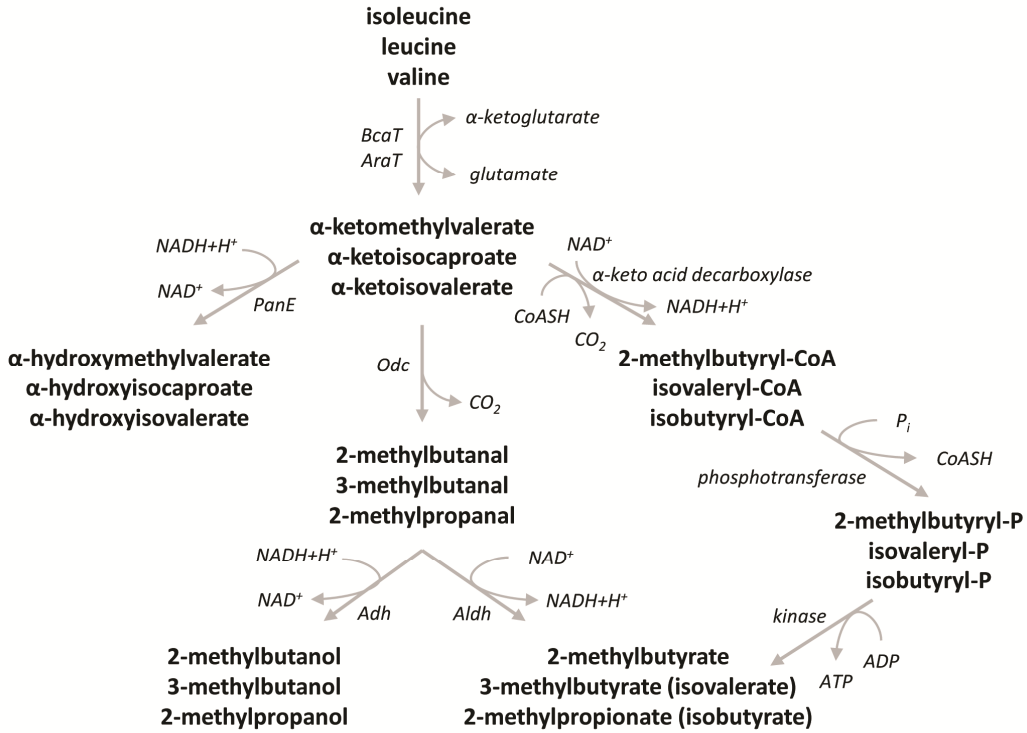
This table is adapted from Yvon & Rijnen (2001), Smit *et al*. (2005), and Singh *et al*. (2003).

4.3.1 Catabolic pathways of branched-chain amino acids

Isoleucine, leucine, and valine are transported into *L. lactis* IL1403 by the major carrier for branched-chain amino acids BcaP (encoded by *ctrA* gene) and by the transporter BrnQ (23, unpublished data). The degradation of amino acids starts by the activity of two aminotransferases, BcaT and AraT (Table 3) to produce the corresponding α-keto acids: α-ketomethylvalerate from isoleucine, α-ketoisocaproate from leucine and α-ketoisovalerate from valine, respectively (Fig. 5). The next step is the conversion of the α-keto acids to α-hydroxy acids (α-hydroxymethylvalerate, α-hydroxyisocaproate, α-hydroxyisovalerate, respectively), aldehydes (2-methylbutanal, 3-methylbutanal, 2-methylpropanal, respectively), and carboxylic acids (2-methylbutyrate, 3-methylbutyrate, 2-methylpropionate, respectively).

The reduction of α-keto acids to α-hydroxy acids derived from branched-chain amino acids is catalyzed by 2-hydroxyisocaproate dehydrogenase (EC 1.1.-) encoded by the *hicD* gene that is widely distributed in LAB. The enzyme belongs to NAD(H)-dependent oxidoreductases and was shown to have the highest activity towards branched-chain α-keto acids (40). The reduction of α-keto acids by *L. lactis* was previously observed when cells were energized with glucose; however, no enzyme has been characterized (76). Lately, PanE present in *L. lactis* IL1403 was described as a 2-hydroxyisocaproate dehydrogenase with the highest catalytic efficiencies for α-ketomethylvalerate, α-ketoisocaproate, and α-ketoisovalerate. PanE belongs to a new family of D -2-hydroxyacid dehydrogenases which is unrelated to the well-described D-2-hydroxyisocaproate

Figure 5. Catabolic pathways for branched-chain amino acids in LAB.



Adh, alcohol dehydrogenase; Aldh, aldehyde dehydrogenase; AraT, aromatic aminotransferase; BcaT, branched-chain aminotransferase; CoASH, coenzyme-A; Odc, α-ketoacid decarboxylase; PanE, 2-hydroxyisocaproate dehydrogenase; P_i, phosphate. The figure was adapted from Fernandez & Zuniga (2006) and Chambellon *et al*. (2009).

dehydrogenase family (14). It was suggested that the physiological role of PanE is to regenerate NAD^+ necessary to catabolize branched-chain amino acids, leading to the production of ATP and aroma compounds (Fig. 5).

The conversion of α-keto acids into aldehydes is catalyzed by α-keto acid decarboxylases (EC 4.1.1.72). The activity of the latter enzyme encoded by the *kdcA* gene has been found only in a

limited number of *L. lactis* strains. The enzyme displayed the highest affinity with α-ketoisovalerate as the substrate and a significantly lower activity towards α-ketomethylvalerate and α-ketoisocaproate (22, 82). The gene is highly homologues to the *ipd* gene that encodes a putative indole pyruvate decarboxylase in *L. lactis* IL1403 (85 % of sequence identity) (10). KdcA activity was never observed in *L. lactis* IL1403 most likely due to a 270-nucleotide deletion at the 3' terminus of the *ipd* gene that produces a non-functional truncated version of the protein (82).

The oxidative decarboxylation of α-keto acids into carboxylic acids derived from branched-chain amino acids catalyzed by α-keto acid decarboxylase, phosphotransferase, and kinase (Fig. 5) appears to be uncommon in LAB (30). Recently, production of 2-methylbutyrate (carboxylic acid of isoleucine) from leucine via a long alternative metabolic route was demonstrated during carbon starvation of *L. lactis* (31). A similar pathway from leucine yielding isovalerate (carboxylic acid of leucine) was described in *L. lactis* subsp. *cremoris* TIL46 (14). Most of the enzymes involved in both routes have not been characterized yet, but both pathways suggest a functional alternative oxidative decarboxylation pathway in *L. lactis* IL1403.

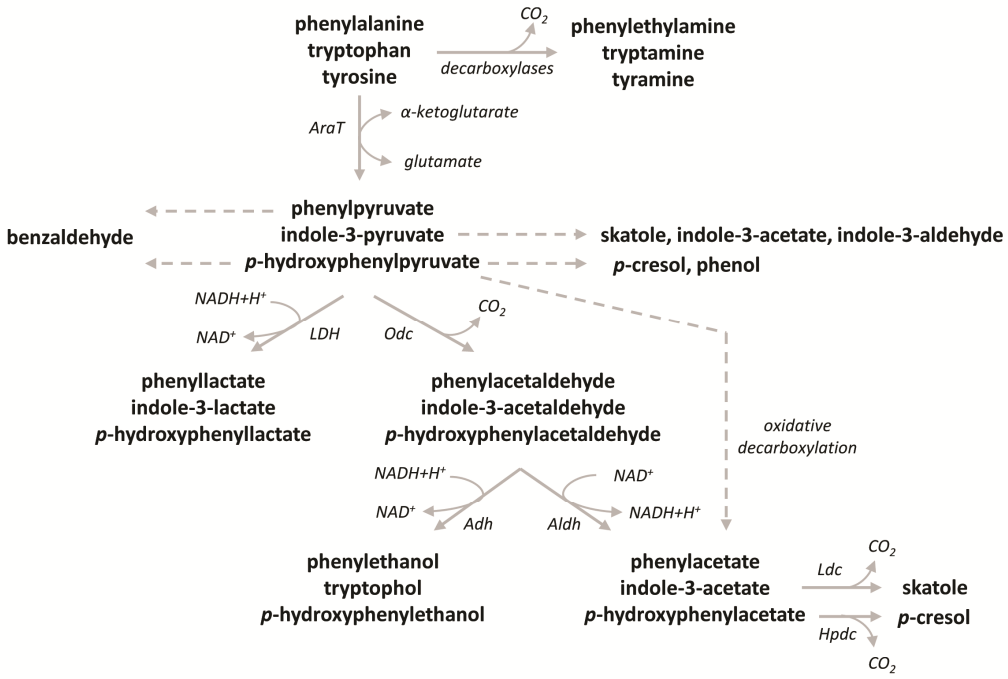
4.3.2 Catabolic pathways of aromatic amino acids

Little is known about the transport system(s) of aromatic amino acids in LAB. Pmf-driven uptake of phenylalanine, tyrosine, and tryptohan by AroT encoded by the *Llmg_2011* gene was recently demonstrated in *L. lactis* subsp. *cremoris* MG1363 (unpublished data). A homologues protein (96 % of sequence identity) is present in *L. lactis* IL1403. Aromatic amino acids can be degraded by transamination or decarboxylation (Fig. 6). Transamination is catalyzed by the AraT aminotransferase (Table 3) and leads to formation of phenylpyruvate, indole-3-pyruvate, and *p*-hydroxyphenylpyruvate from phenylalanine, tryptophan, and tyrosine, respectively. Transamination catalyzed by AraT plays an essential role in the biosynthesis of phenylalanine and tyrosine in *Lactococcus* sp., but is not essential for tryptophan biosynthesis due to the presence of an alternative pathway (6).

α-Keto acids formed from aromatic amino acids are further degraded by enzymatic reactions into α-hydroxy acids (phenyllactate, indole-3-lactate, *p*-hydroxyphenyllactate) by dehydrogenation, to aldehydes (phenylacetaldehyde, indole-3-acetaldehyde, *p*-hydroxyphenylacetaldehyde) by decarboxylation, or to carboxylic acids (phenylacetate, indole-3-acetate, *p*-hydroxyphenylacetate) by oxidative decarboxylation. Most of the enzymes involved in flavor development from aromatic amino acids have not been characterized. Next to enzymatic reactions, various spontaneous chemical conversions are possible (Fig. 6) (30, 83, 99).

α-Hydroxy acids are formed by activity of NAD(H)-dependent 2-hydroxyacid dehydrogenase. Formation of phenyllactate from phenylpyruvate observed in *L. lactis* subsp. *cremoris* NCDO763 and *Lactobacillus plantarum* is catalyzed by L-lactate dehydrogenase (14, 42, 97) that is also

Figure 6. Catabolic pathways for aromatic amino acids in LAB.



Adh, alcohol dehydrogenase; Aldh, aldehyde dehydrogenase; AraT, aromatic aminotransferase; Hpdc, hydroxyphenylacetate decarboxylase; Ldc, indole-3-acetate decarboxylase; LDH, L-lactate dehydrogenase; Odc, α-ketoacid decarboxylase. Dashed lines indicate chemical reactions. The figure was adapted from Fernandez & Zuniga (2006).

found in *L. lactis* IL1403 (10).

Oxidative decarboxylation of aromatic α-keto acids phenylpyruvate, indole-3-pyruvate, and *p*-hydroxyphenylpyruvate has been shown to result in formation of significant amounts of phenylacetate, indole-3-acetate, and *p*-hydroxyphenylacetate in different *L. lactis* strains. Intermediates of the decarboxylation pathway, i.e phenylacetaldehyde, indole-3-acetaldehyde, and *p*-hydroxyphenylacetaldehyde, were not identified (97). The reduction of aldehydes into alcohols has never been demonstrated in LAB (30). The enzymatic conversion of indole-3-acetate to skatole and *p*-hydroxyphenylacetate to *p*-cresol was reported only in some strains of lactobacilli (37, 95).

Chemical conversions producing *p*-cresol, skatole, benzaldehyde, phenylethanol, idole-3-acetate, and indole-3-aldehyde have been reported in different LAB among which *Lactococcus* sp. (66). The formation of benzaldehyde is catalyzed in the presence of several divalent metal ions (70).

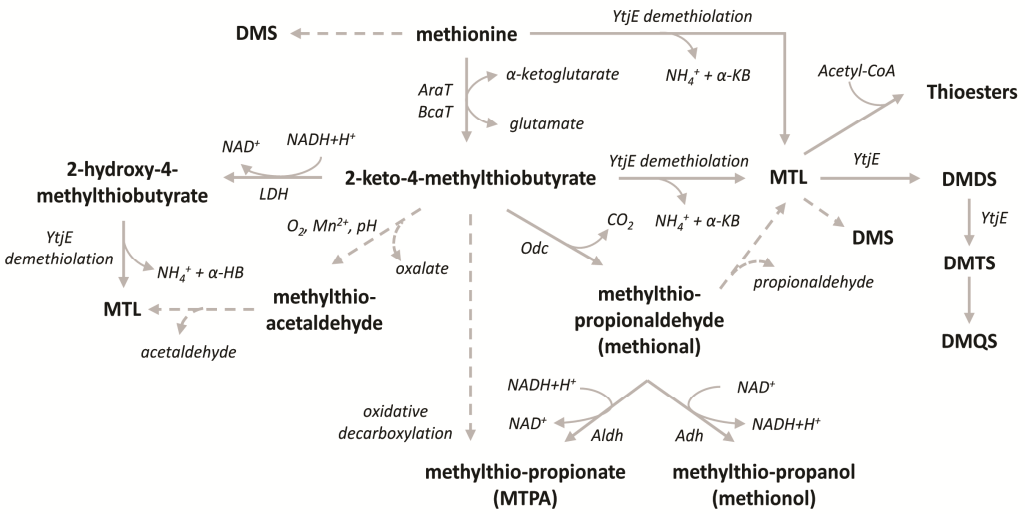
Production of biogenic amines from aromatic amino acids by decarboxylation (Fig. 6) is not common in *L. lactis*. The production of tyramine was observed in *L. lactis* IPLA655, in which the

tyrosine-tyramine gene cluster was found. The cluster consists of the genes encoding a putative tyrosyl tRNA-synthetase, a tyrosine decarboxylase (*tdcA*), and a tyrosine-tyramine exchanger (29). The same or other clusters related to the production of aromatic biogenic amines are not found in *L. lactis* IL1403 (10).

4.3.3 Catabolism of methionine

The methionine uptake system in *L. lactis* IL1403 is encoded by the *plpABCD-ydcBD* transcriptional unit (10, 30). Also, the secondary transporter BcaP can transport methionine to some extend (23). Methionine metabolism follows two pathways that convert sulfur-containing amino acids into aroma compounds (Fig. 7). The first one is simultaneous deamination and demethiolation of methionine into methanethiol (MTL) that is catalyzed by methionine lyase (30,

Figure 7. Catabolic pathways for methionine described in LAB.



Adh, alcohol dehydrogenase; Aldh, aldehyde dehydrogenase; AraT, aromatic aminotransferase; BcaT, branched-chain aminotransferase; DMDS, dimethyl disulfide; DMQS, dimethyl tetrasulfide; DMS, dimethyl sulfide; DMTS, dimethyl trisulfide; LDH, lactate dehydrogenase; MTL, methanethiol; Odc, α-ketoacid decarboxylase; α-HB, α-hydroxybutyrate; α-KB, α-ketobutyrate; YtjE, C-S lyase. Dashed lines indicate chemical reactions. The figure was adapted from Landaud *et al*. (2008) and Martinez *et al*. (2006).

51). Recently, YtjE, a C-S lyase that exhibits α, γ-elimination activity, was isolated from *L. lactis* IL1403. The enzyme catalyzes the formation of methanethiol from methionine, 2-hydroxy-4-methylthiobutyrate (α-hydroxy acid of methionine), and 2-keto-4-methylthiobutyrate (α-keto acid from methionine). MTL is further converted into dimethyl disulfide (DMDS) and dimethyl trisulfide (DMTS) by the same enzyme (63).

The second pathway is transamination of methionine to 2-keto-4-methylthiobutyrate that is catalyzed by aminotransferases BcaT and AraT (Fig. 7, Table 3) (75, 97, 98). 2-keto-4-methylthiobutyrate is an important intermediate that can be further converted via many routes. Formation of 2-hydroxy-4-methylthiobutyrate (α-hydroxy acid of methionine) is catalyzed by L-lactate dehydrogenase at the expense of NADH (14). The α-hydroxy acid can be further metabolized to methanethiol by the activity of YtjE that was mentioned above (63). Decarboxylation of the α-keto acid of methionine to methional has been reported in one strain of *L. lactis*, but the responsible enzyme was not characterized (1). In some LAB the reaction is catalyzed by KdcA, the α-keto acid decarboxylase specific for branched-chain amino acids (82); however, an active form of this enzyme is not present in IL1403 (see above) (10). Enzymes responsible for methional oxidation or reduction to methylthio-propionate or methionol, respectively, have not been identified in *Lactococcus* sp.. In some LAB, methylthio-propionate is formed by chemical or enzymatic oxidative decarboxylation of 2-keto-4-methylthiobutyrate. An additional source of methanethiol is provided by chemical conversion of methional or 2-keto-4-methylbutyrate via methylthioacetaldehyde (51).

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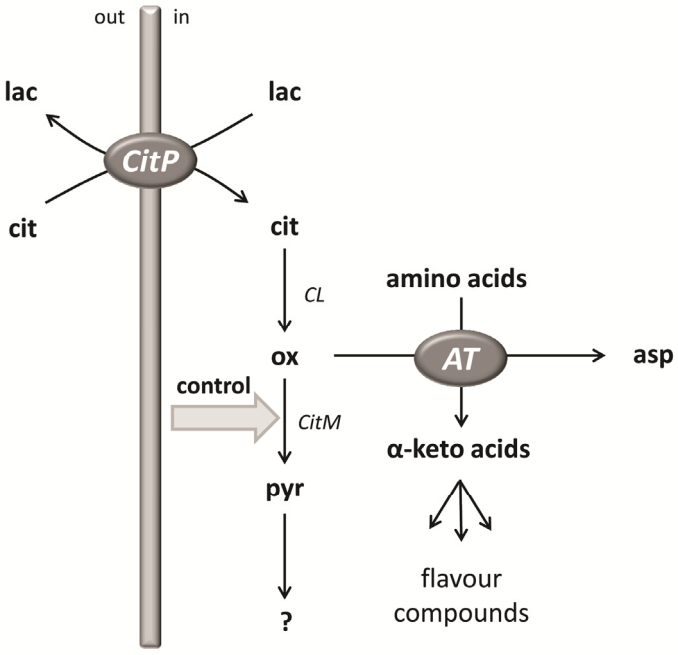
Outline of the thesis

1

Lactococcus lactis is currently the most extensively studied and the best characterized organism among all LAB. The well-established status of *L. lactis* as a cheese starter bacterium, and therefore, food organism, its well-known and relatively simple physiology, the complete genome sequence of 7 strains, and the development of several genetic tools make the organism a suitable target for metabolic engineering strategies aimed at the improvement of food quality. Successful example of metabolic engineering is the rerouting of lactococcal pyruvate metabolism to products other than lactic acid, such as the aroma compound diacetyl (69). The aim of the study described in this thesis was to engineer a citrate driven transamination pathway in *L. lactis* to produce flavor compounds important in dairy industry derived from amino acid catabolic pathway (Fig. 8). Transamination, the first step in the conversion of amino acids, is often limited by lack of an efficient source of an α-keto donor (83, 91, 99). Citrate fermentation provides an alternative source of an α-keto acid in the form of oxaloacetate. The internal pool of oxaloacetate is controlled by oxaloacetate decarboxylase that provides a control point to redirect citrate metabolism towards transamination. Rerouting of oxaloacetate produced from citrate to transamination rather than to decarboxylation allows for improved production of aroma compounds.

Figure 8. Scheme representing production of flavor compounds by citrate driven transamination in *L. lactis* IL1403.



Oxaloacetate decarboxylase provides a control point to redirect citrate metabolism towards transamination (arrow). asp, aspartate; AT, aminotransferase; cit, citrate; CitM, oxaloacetate decarboxylase; CitP, citrate permease; CL, citrate lyase; lac, L-lactate; ox, oxaloacetate; pyr, pyruvate.

Chapter 2 describes the characteristics of the citrate metabolic pathway in *L. lactis* IL1403(pFL3), the strain that later on was used to engineer the pathway. The strain contains the citrate uptake system CitP encoded on a plasmid and the citrate metabolic enzymes encoded on the chromosome. As expected, cometabolism of citrate and the carbohydrate glucose by resting cells resulted in the formation of the flavor compound acetoin and a proton motive force consisting of both a membrane potential and a transmembrane pH gradient was generated efficiently. The same result was obtained when glucose was replaced by L-lactate which is explained by the so-called L-lactate shuttle mechanism that will play an important role in the studies described in the next chapters. New insights in the citrate fermentation pathway followed from the study of the metabolism of citrate in the absence of glucose or L-lactate that resulted in a shift of the product profile and different energetic properties. Analysis of the data revealed a splitting of the pathway following pyruvate yielding the end product acetate rather than acetoin, a pathway that does not generate proton motive force. The intermediate accumulation of pyruvate and α-acetolactate in the medium revealed the ability of the citrate transporter to take up citrate into the cells in exchange with these intermediates of the pathway instead of in exchange with L-lactate. The promiscuity of CitP is crucial to the understanding of the pathway in the oxaloacetate deficient mutant described in Chapter 3. The studies in Chapter 2 resolved the operative modes of the citrate metabolic pathway in *L. lactis* under different conditions and showed that controlling oxaloacetate decarboxylase to overproduce cytoplasmic oxaloacetate is crucial.

Chapter 3 describes the metabolic breakdown of citrate in the oxaloacetate decarboxylase deficient mutant derived from strain IL1403(pFL3) and elaborates on the metabolic response of *L. lactis* during citrate fermentation when oxaloacetate accumulates in the cytoplasm. Surprisingly, the mutant strain showed nearly identical rates of citrate consumption compared to the parent strain both in the presence of a carbohydrate and in the absence, but under both conditions acetate was the end product and no proton motive force was generated. Citrate uptake was coupled to the excretion of oxaloacetate with a high yield of 80-85 % suggesting citrate/ oxaloacetate exchange. The substrate specificity of CitP towards oxaloacetate was confirmed by studies with the parental strain IL1403(pFL3) containing CitP, which consumed oxaloacetate and the original strain IL1403 not containing CitP, which did not. Citrate fermentation in the mutant proceeds in two steps. During the first step, oxaloacetate inadvertently accumulates in the cytoplasm and the physiological response of *L. lactis* is to excrete oxaloacetate in exchange with citrate by an electroneutral mechanism catalyzed by CitP. Subsequently, in a second step, oxaloacetate is taken up by CitP and metabolized slowly to pyruvate by a cryptic decarboxylase and further to acetate as was described in Chapter 2. Most importantly, the studies described in Chapter 3 show that in the oxaloacetate decarboxylate deficient mutant, oxaloacetate accumulates in the cytoplasm, a prerequisite for driving citrate metabolism into the transamination route that will be described in Chapter 5.

Chapter 4 elaborates on the observation made in Chapters 2 and 3 that a wide range of substrates are exchanged with citrate during fermentation in *L. lactis*, i.e. L-lactate during citrate/ glucose cometabolism and the metabolic intermediates/end products pyruvate, α-acetolactate, and/or acetate when no L-lactate is present, and oxaloacetate in the mutant strain. A systematic study of the substrate specificity of CitP is presented using an assay based on the 'shuttle mechanism' described in Chapter 2. The binding and translocation properties of CitP were analyzed systematically for a wide variety of mono- and dicarboxylates of the form $X\text{-}CR_2\text{-}COO^-$, in which X represents OH (2-hydroxy acid, f.i. L-lactate or α-acetolactate), O (2-keto acid, f.i. oxaloacetate or pyruvate), or H (acid, f.i. acetate) and R groups that differ in size, hydrophobicity, and composition. It is demonstrated that CitP is a very promiscuous carboxylate transporter. The broad substrate specificity is discussed in the context of a model of the binding site of CitP. Important for the main topic of the thesis, many of the substrates of CitP are intermediates or products of amino acid metabolism such as α-keto acids produced during transamination and further metabolites like α-hydroxy acids suggesting that CitP may have a broader physiological function than in citrate fermentation alone as will be discussed in Chapter 5 and 6.

Chapter 5 demonstrates the main goal of the thesis. Metabolic engineering of *L. lactis* to reroute the citrate metabolism into transamination by knocking-out oxaloacetate decarboxylase is efficient and can improve the quality of food fermented products. It is demonstrated that oxaloacetate which accumulates in the cytoplasm of the mutant strain at high concentration (described in Chapter 3) is successfully redirected into transamination. The strain produced high amounts of the corresponding α-keto acids in the presence of citrate and the branched-chain amino acids, aromatic amino acids, and methionine. The study demonstrates that transaminases of *L. lactis* accept oxaloacetate as keto donor. In addition to excretion of oxaloacetate, the cells respond to the oxaloacetate stress caused by the mutation by upregulation of the transaminases in the cytoplasm. The α-keto acids and downstream products are known substrates of CitP as was demonstrated in Chapter 4 and during citrate driven transamination in *L. lactis* these flavor compounds may be excreted from the cell by CitP in exchange with the precursors of the pathway, citrate and oxaloacetate.

Chapter 6, finally, solves an old enigma in the production of flavor compounds in the food industries. While it is known that α-ketoglutarate is the best keto donor for lactococcal transaminases, addition to the medium does not seem to result in higher production of flavor compounds. The studies in Chapter 6 show that the limitation is in the lack of an uptake system for α-ketoglutarate. Importantly, it is demonstrated that the citrate transporter CitP efficiently transports α-ketoglutarate allowing strains that express CitP to produce flavor compounds from amino acids by adding external α-ketoglutarate. The rate of flavor compounds production driven by α-ketoglutarate transamination was enhanced by coupling to carbohydrate fermentation, conditions under which further metabolism of α-keto acids into α-hydroxy acids was observed. The studies described in Chapters 5 and 6 suggest that the physiological role of CitP in LAB is

much broader than citrate uptake in the citrate fermentation pathway and the transporter may play an important role in development of flavor compounds driven by citrate or α-ketoglutarate transamination.

Chapter 2

Citrate uptake in exchange with intermediates of the citrate metabolic pathway in *Lactococcus lactis* IL1403

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2

Abstract

Carbohydrate/citrate cometabolism in *Lactococcus lactis* results in the formation of the flavor compound acetoin. Resting cells of strain IL1403(pFL3) rapidly consumed citrate while producing acetoin when substoichiometric concentrations of glucose or L-lactate were present. A proton motive force was generated by electrogenic exchange of citrate and lactate catalyzed by the citrate transporter CitP and proton consumption in decarboxylation reactions in the pathway. In the absence of glucose or L-lactate, citrate consumption was biphasic. During the first phase hardly any citrate was consumed. In the second phase, citrate was converted rapidly, but without the formation of acetoin. Instead, significant amounts of the intermediates pyruvate and α-acetolactate, and the end product acetate, were excreted from the cells. It is shown that the intermediates and acetate are excreted in exchange with the uptake of citrate catalyzed by CitP. The availability of exchangeable substrates in the cytoplasm determines both the rate of citrate consumption and the end product profile. It follows that citrate metabolism in *L. lactis* IL1403 (pFL3) splits up in two routes after the formation of pyruvate, one the well characterized route yielding acetoin, and the other, a new route yielding acetate. The flux distribution between the two branches changes from 85/15 in the presence of L-lactate to 30/70 in the presence of pyruvate. The proton motive force generated was highest in the presence of L-lactate and zero in the presence of pyruvate suggesting that the pathway to acetate does not generate proton motive force.

Introduction

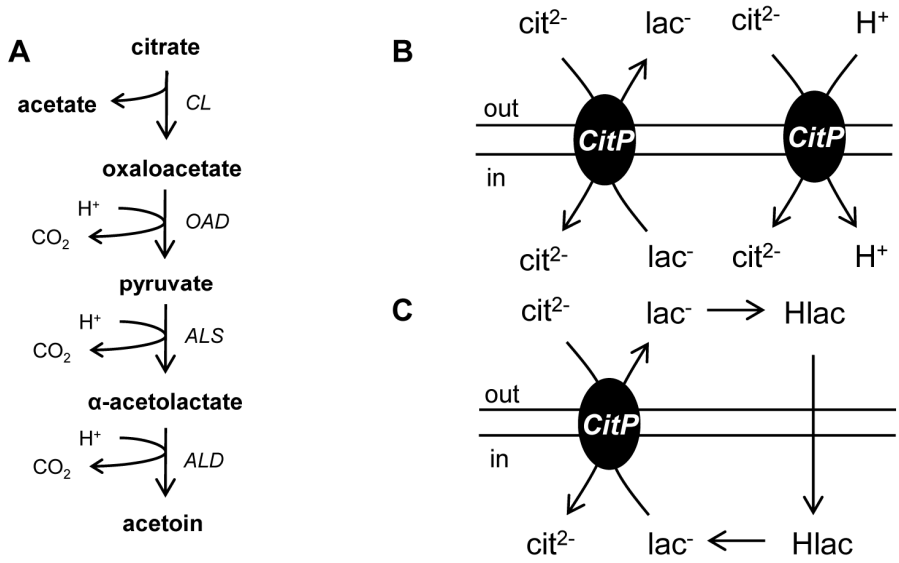
Citrate fermentation is a strain specific trait among Lactic Acid Bacteria (LAB) that is associated with the production of carbon dioxide and C4-flavour compounds (17). During carbohydrate/citrate cometabolism, additional pyruvate from citrate added to the central pyruvate pool in the glycolytic pathway, is converted to acetoin (Fig. 1A). Citrate is transported into the cell by the secondary transporter CitP. Inside, citrate is converted to acetate and oxaloacetate catalyzed by citrate lyase. Acetate leaves the cell, while oxaloacetate is decarboxylated to pyruvate by a soluble oxaloacetate decarboxylase (36). α-Acetolactate synthase converts two molecules of pyruvate to one molecule of α-acetolactate while releasing carbon dioxide. The majority of α-acetolactate is decarboxylated to acetoin by α-acetolactate decarboxylase. A small part of the chemically unstable α-acetolactate results in the formation of diacetyl in a non-enzymatic oxidative decarboxylation reaction (16, 33). Citrate fermenting LAB strains are valuable for use as starters in the dairy and wine industries since compounds such as carbon dioxide, acetoin, and diacetyl promote the organoleptic properties of fermentation products.

Citrate metabolism in LAB is a metabolic energy generating pathway. The pathway generates an electrochemical gradient of protons (proton motive force, pmf) across the cell membrane (5, 27, 28) by a secondary mechanism in which membrane potential and pH gradient are generated in separate steps (21, 22). The transporter CitP catalyzes uptake of divalent citrate in exchange for monovalent lactate, which results in a membrane potential of physiological polarity (i.e. inside negative) (Fig. 1B). The pH gradient (inside alkaline) is the result of proton consumption in the decarboxylation reactions taking place in the cytoplasm. The pathway functions as an indirect proton pump.

Usually, secondary pmf generating pathways are simple pathways built around a single decarboxylation reaction. A carboxylate substrate is taken up by a transporter, decarboxylated in the cytoplasm, and then the decarboxylation product is excreted by the same transporter in an exchange process. Well-studied examples are malate decarboxylation in *Lactococcus lactis* (malolactic fermentation) (32), oxalate decarboxylation in *Oxalobacter formigenes* (3), and catabolic amino acid decarboxylation pathways (e.g. 2, 18, 30, 41, 44). Typically, the transporters in the pathways, take up the substrate in exchange with the product of the pathway and are termed precursor/product exchangers (31). Remarkably, in the citrate fermentation pathway in LAB, citrate is taken up in exchange with lactate (citrolactic fermentation), which is not the product of the citrate degradation pathway but of glycolysis.

Following the discovery of citrate fermentation as a secondary proton motive force generating pathway in *Leuconostoc mesenteroides* (27, 28) and subsequent confirmation in *L. lactis* (26), the citrate transporter CitP was extensively characterized by transport studies in membrane vesicles (4, 5, 6, 27). The transporter is a member of the 2-hydroxycarboxylate transporter (2HCT) family (40) (TC classification 2.A.24 (34)) in which also are found the malate/lactate exchanger MleP that functions in the malolactic fermentation pathway in LAB (32) and the Na^+-citrate symporter CitS

Figure 1. Citrate metabolism in LAB.



A. Citrate fermentation pathway yielding acetoin. Enzymes: CL, citrate lyase; OAD, oxaloacetate decarboxylase; ALS, α-acetolactate synthase; ALD, α-acetolactate decarboxylase. The stoichiometry of the reactions was not taken into account. B. Kinetic modes of the citrate transporter CitP. Left, exchange (fast) of external citrate (cit^{2-}) and internal L-lactate (lac^{-}), right, unidirectional uptake (slow) of citrate (cit^{2-}) in symport with a H^{+}. C. L-lactate shuttle mechanism. L-lactate added to the outside of the cells, allows CitP to operate in the fast cit^{2-}/lac^{-} exchange mode by reentering the cell in the permeative protonated state. The net result is the uptake of cit^{2-} and a H^{+}.

that functions in citrate fermentation in *Klebsiella pneumoniae* (12). The name of the family refers to the 2-hydroxycarboxylate (2-HC) motif, i.e. $HO\text{-}CR_2\text{-}COO^{-}$, shared by the substrates of the transporters in the family (4). Kinetic studies of CitP revealed two modes of transport, symport of divalent citrate with one proton and exchange of divalent citrate with monovalent lactate (Fig. 1B) (25, 27, 35). Since the former was much slower than the latter, it was concluded that CitP is a symporter that was optimized to catalyze exchange, which would be the physiological function. Chase studies with membrane vesicles loaded with radiolabeled citrate showed a remarkable tolerance of CitP (and MleP, but not CitS) to the R substituents of 2-hydroxycarboxylate substrates. CitP accepted R groups differing in size and charge, ranging from H atoms in glycolate to acetyl groups in citrate, and everything in between in physiological or non-physiological substrates. The specificity of CitP for substrates carrying different charges forms the mechanistic basis for membrane potential generation (4, 6).

In this study, the impact of the broad substrate specificity of CitP on the metabolism of citrate in *L. lactis* IL1403 is demonstrated. It is shown that the metabolic intermediates pyruvate and α-acetolactate and the end product acetate can function in the exchange reaction catalyzed by CitP to take up citrate. The availability of exchangeable metabolites in the cytoplasm determines the end product profile of the pathway. Unexpectedly, following the conversion of citrate to pyruvate, the pathway branched into a route to acetate in addition to the one to acetoin.

Materials and methods

Materials

M17 broth was obtained from Merck, Darmstadt, Germany. Citrate lyase (Cat.No.10354074001), L-malate dehydrogenase (Cat.No.10127256001), and L-lactate dehydrogenase (Cat.No.10127230001) were obtained from Roche Applied Science. Ethyl 2-acetoxy-2-methylacetoacetate, α-naphtol, and creatine were obtained from Sigma Aldrich, acetoin, diacetyl, and 2,3-butanediol from Fluka. The 3,3'-dipropylthiocarbocyanine iodide ($DiSC_3$) and 2',7'-bis-(2-carboxyethyl)-5(and-6)-carboxyfluorescein (BCECF, acid form) probes were obtained from Invitrogen Molecular Probes.

Bacterial strain and growth condition

Lactococcus lactis IL1403(pFL3) harbouring the pFL3 plasmid containing the *citP* gene under the control of the *Streptococcus pneumoniae polA* promoter was used in this study (24). Cells were grown in M17 medium supplemented with 0.5 % (w/v) glucose (M17G) and 5 µg/ml tetracycline in 100 ml serum bottles without agitation at 30 ^{o}C. Initial pH of the medium was adjusted to 7.0 by the addition of HCl or NaOH. Growth was followed by measuring the turbidity at 660 nm. In the middle of exponential phase, cells were harvested by spinning for 10 min at 3000 rpm when the culture reached a turbidity of 0.6. The pellets were washed two times with 50 mM potassium phosphate pH 5.8 buffer, and finally, were resuspended in the same buffer.

Citrate consumption by resting cells

The assay was performed in a total volume of 1.5 ml. Resting cells at an OD_{660} of 1.5 in 50 mM potassium phosphate pH 5.8 buffer were incubated at 30 ^{o}C without agitation for 10 min. At t=0, citrate was added at a concentration of 2 mM together with glucose, L-lactate, pyruvate, 2-hydroxyisobutyrate (2-HIB), α-acetolactate or acetate at the indicated concentrations. α-Acetolactate was prepared freshly by saponification of ethyl 2-acetoxy-2-methylacetoacetate (diester of α-acetolactate) according to (13). 10 µl of ethyl 2-acetoxy-2-methylacetoacetate was added to 1 ml of 180 mM NaOH at 10 ^{o}C for 20 min in a stirred closed tube. Then, 2 ml of 100 mM maleate pH 6.0 buffer and 0.35 ml of 100 mM maleic acid or 2.35 ml potassium phosphate pH 5.8 buffer were added. The final solution was 15.4 mM of α-acetolactate containing equimolar concentrations of acetate and ethanol in maleate pH 6.0 buffer or potassium phosphate pH 5.8 buffer. Samples of 100 µl of the cell suspensions were taken every 5 min and immediately centrifuged for 0.5 min at maximum speed in a table top centrifuge. The supernatant was stored on ice until further analysis by the enzymatic assays and/or HPLC (see below). Measurements of the concentrations of citrate, oxaloacetate, and pyruvate were in good agreement between the two methods.

Enzymatic assays

Citrate, oxaloacetate, and pyruvate were measured using the commercially available enzymes citrate lyase, L-malate dehydrogenase, and L-lactate dehydrogenase. The assay was performed in microtiter plates. An aliquot of 30 µl of the supernatant was added to 50 mM glycyl-glycine pH 7.8 buffer, giving a total volume of 200 µl, containing 0.45 mM NADH and 0.95 U of L-lactate dehydrogenase. Pyruvate in the sample is converted to L-lactate at the expense of NADH. Oxaloacetate in the same sample was measured after addition of 1 µl 1.9 U of L-malate dehydrogenase, resulting in the conversion of oxaloacetate to L-malate at the expense of NADH. Subsequent addition of 1 µl 0.032 U of citrate lyase converts citrate in the sample to oxaloacetate (and pyruvate), resulting in an additional decrease in the NADH concentration equivalent to the citrate concentration present in the samples. The decrease in NADH concentration was measured spectroscopically at 340 nm.

Colorimetric determination of acetoin and diacetyl

Acetoin and diacetyl were measured according to (43). Acetoin and diacetyl react with guanido groups of creatine in alkaline medium to give a pink colour. A volume of 30 µl of the supernatant was mixed with 10 µl of 0.5 % (w/v) creatine, 10 µl of 5 % (w/v) α-naphtol freshly dissolved in 2.5 M NaOH, and 150 µl of water. Colour development was measured at OD_{530} after 5 min and after 40 min of incubation in the dark at room temperature to determine diacetyl and acetoin, respectively. The amount of acetoin was calculated from a calibration curve of known concentrations. Only trace amount of diacetyl were measured in the supernatants indicating negligible spontaneous non-enzymatic oxidation of α-acetolactate to diacetyl.

HPLC analysis

An aliquot of 10 µl of the supernatants was loaded on an Aminex HPX-87H anion-exchange column (Bio-Rad Laboratories, Inc., Richmond, CA) operated at 30 ^{o}C in isocratic mode, using 0.005 M H_2SO_4 as the mobile phase, and a flow rate of 0.8 ml/min. The samples were analyzed for the presence of 2,3-butanediol, acetate, acetoin, α-acetolactate, citrate, diacetyl, ethanol, formate, isocitrate, α-ketoglutarate, L-lactate, oxaloacetate, and pyruvate. α-Acetolactate was prepared freshly as described above. Concentrations were estimated from standard curves prepared separately for all these compounds and reported as averages from three independent measurements with standard deviations.

Measurement of internal pH (ΔpH) and membrane potential (ΔΨ)

Cells were prepared as described above. Resting cells resuspended to high density (typically containing 50 mg/ml of protein) in 50 mM potassium phosphate pH 5.8 buffer were loaded with BCECF by mixing 20 µl of the cell suspension with 1 µl of a 10 mM BCECF solution and 0.5 µl of 0.5 M HCl. The suspension was incubated at room temperature for exactly 5 min, after which 1 ml

of 50 mM potassium phosphate pH 5.8 buffer was added. The cells were washed 4 times, resuspended in 200 µl of buffer, and kept on ice until use. Fluorescence measurements were performed in a 1 cm cuvette containing 3 ml 50 mM potassium phosphate pH 5.8 buffer equilibrated at 30 ^{o}C and 5-10 µl of BCECF loaded cells, depending upon the concentration of BCECF in the cells. The cuvette was stirred with a magnetic stirring bar. The excitation and emission wavelengths were 502 and 525 nm with slit widths of 4 and 16 nm, respectively. The fluorescence signal was sampled every second. The opening of the measurement compartment caused loss of data during the first 5-6 seconds after an addition of substrate to the cuvette. The cytoplasmic pH was calculated as described (29).

The membrane potential was measured qualitatively with the fluorescent probe $DiSC_3$ (37). Decrease in fluorescence intensity correlates with an increase in electrical potential across the membrane. A volume of 5-10 µl of the cells, resuspended to an OD_{660} of 1.5 in 50 mM potassium phosphate pH 5.8 buffer, was added to 3 ml of the same buffer in a 1 cm cuvette. $DiSC_3$ was added from a stock solution in absolute ethanol to a final concentration of 2 µM and the system was left to equilibrate for 5 min at 30 ^{o}C. Adding the same volume of absolute ethanol did not affect the citrate consumption rate of the cells. Fluorescence measurements were performed using excitation and emission wavelengths of 500 and 705 nm, respectively, and slit widths of 8 nm.

Results

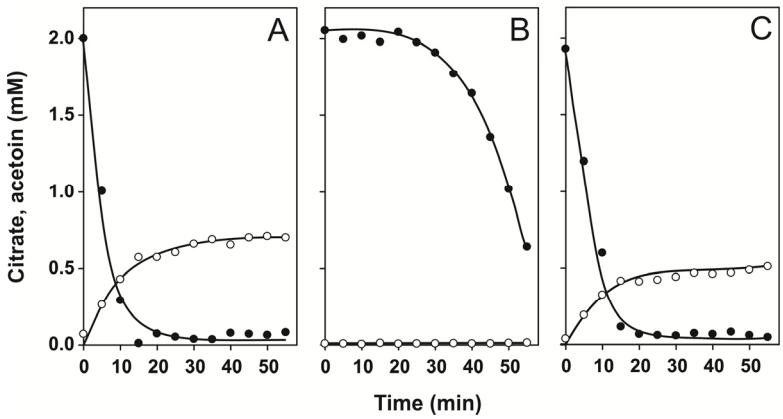
Citrate metabolism by L. lactis IL1403(pFL3)

Citrate consumption by resting cells of *L. lactis* IL1403(pFL3) was studied in the presence and absence of glucose. Strain IL1403 encodes all citrate metabolic enzymes on the chromosome except for the citrate transporter, CitP (8). The latter is expressed constitutively from the plasmid pFL3 (24). The strain was grown in M17 broth medium supplemented with glucose until the mid-exponential growth phase (OD_{660}=0.6). In the presence of glucose, resting cells resuspended to an OD_{660} of 6 in 50 mM potassium phosphate pH 5.8 buffer rapidly consumed citrate and at the same time acetoin was produced (Fig. 2A). Diacetyl was not detected as a product in any significant quantity. Glucose at a concentration of 0.1 mM was converted to 0.2 mM of L-lactate at the 5 min time point after which the L-lactate concentration remained constant during the remainder of the experiment. At a 20 times higher concentration (2 mM), citrate was consumed at a rate of 0.18 mM/min that was constant down to citrate concentrations of about 0.2 mM, indicating a high affinity of the pathway for citrate (zero order kinetics). Acetoin production followed citrate consumption in time at a rate of 0.05 mM/min. When all citrate was consumed, 0.67 mM of acetoin was produced giving a yield of almost 70 % when taking into account that the formation of one molecule of acetoin requires two molecules of citrate. Acetoin was exclusively produced when citrate was present; no acetoin was produced from glucose in the absence of

citrate (not shown). Following full conversion of citrate, the concentration of acetoin slowly increased suggesting accumulation of a precursor of acetoin during citrate metabolism (Fig. 2A).

In the absence of glucose, citrate metabolism by the same resting cells of *L. lactis* IL1403(pFL3) showed two phases (Fig. 2B). A first phase, during which no significant consumption of citrate was observed, was followed by a phase, in which citrate was consumed at a rate of 0.08 mM/min, which is about half the rate observed in the presence of glucose. Remarkably, no acetoin was produced. These results will be discussed in the Discussion section.

Figure 2. Citrate metabolism in *L. lactis* IL1403(pFL3).



A concentration of 2 mM of citrate was added to resting cells in the presence of 0.1 mM glucose (A), no further additions (B), and 0.2 mM L-lactate (C). The citrate (●) and acetoin (○) concentrations in the supernatant were measured at the indicated time points.

Exchange and unidirectional transport catalyzed by CitP

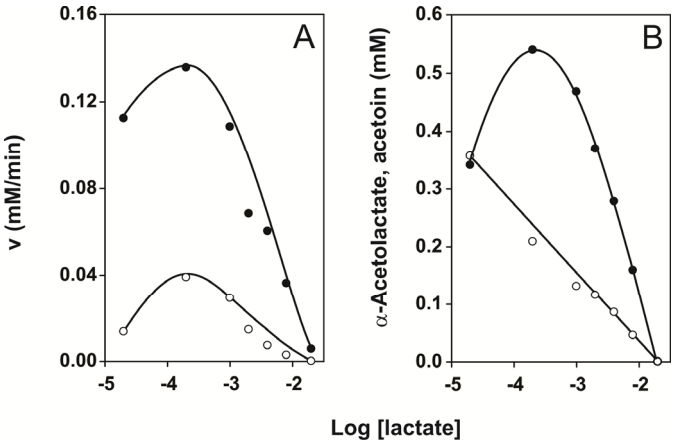
The citrate transporter CitP catalyzes both unidirectional transport of citrate in symport with a proton and bidirectional transport of citrate in exchange with the metabolic end product L-lactate (Fig. 1B). Exchange is catalyzed much more efficiently than symport (4, 27). The properties of CitP suggest that in the presence of glucose citrate is taken up rapidly in exchange with L-lactate produced by glycolysis and that the first phase observed in the absence of glucose represents slow unidirectional citrate uptake (Fig. 2). Subsequently, the second fast phase would represent an exchange mode in which citrate is taken up in exchange with a metabolic product formed from citrate.

Citrate metabolism by resting cells in the absence of glucose, but in the presence of L-lactate added to the buffer, resulted in a fast consumption of citrate (Fig. 2C). At a concentration of 0.2 mM L-lactate, the rate was 0.17 mM/min, similar as in the presence of 0.1 mM glucose (Fig. 2A). The yield of acetoin produced from citrate in the presence of L-lactate was a bit lower than the amount produced when glucose was present, 0.5 and 0.67 mM, respectively. Again, no significant

amounts of diacetyl were formed. Similarly as observed in the presence of glucose, the concentration of acetoin increased slowly following full conversion of citrate (Fig. 2C).

A 10-fold lower concentration of L-lactate added to the buffer or produced from glucose supported rapid metabolism of 2 mM citrate (Fig. 2A, C) which is explained by the 'lactate shuttle' depicted in Fig. 1C. L-lactate exported from the cells by CitP reenters as L-lactic acid by passive diffusion (27). The concentration of L-lactate required to catalyze citrate uptake in the fast citrate/L-lactate exchange mode was remarkably low (Fig. 3A). The rate was optimal at a concentration of 0.2 mM and still 80 % of this rate was observed at a concentration as low as 20 µM. Higher concentrations of L-lactate were inhibitory, resulting in complete inhibition at a concentration of 20 mM. The acetoin production rate showed the same profile.

Figure 3. Effect of L-lactate concentration on citrate metabolism in *L. lactis* IL1403(pFL3).

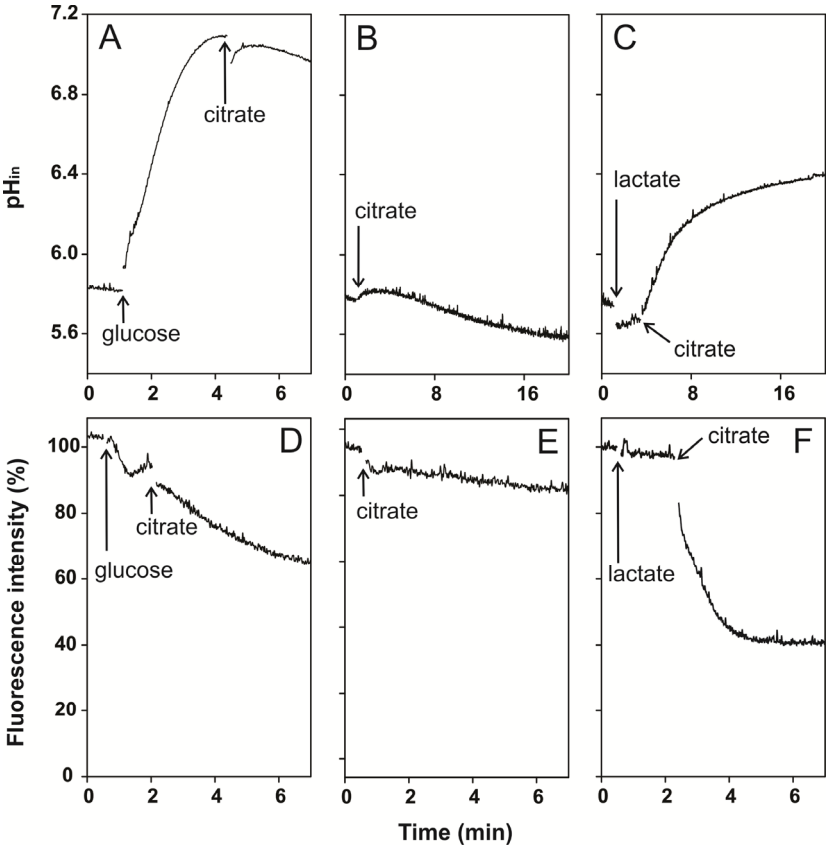


A concentration of 2 mM of citrate was added to resting cells in the presence of L-lactate at concentrations ranging from 0.2 mM to 20 mM. A. Rates of citrate consumption (●) and acetoin production (○). Rates were deduced from the decrease in citrate and increase in acetoin concentrations in the period during which 75% of citrate was consumed, assuming zero-order kinetics (see Fig. 2C). B. Concentrations of acetoin (●) and α-acetolactate (○) produced when all citrate was consumed.

The citrate metabolic pathway in LAB is proton motive force (pmf) generating both in the symport and exchange modes of the transporter (26, 27, 28). The two components of the pmf, pH gradient (ΔpH) and membrane potential (ΔΨ), generated under the experimental conditions above, were measured with the fluorescent dye BCECF (29) and the potentiometric probe, 3,3'-dipropylthiadicarbocyanine iodide ($DiSC_3$) (37), respectively. The cytoplasmic pH of the resting cells was very close to the pH of the buffer (Fig. 4). In the presence of 0.1 mM of glucose, a significant pmf was generated, presumably the result of proton pumping by F_0F_1-ATPase driven by hydrolysis of ATP produced in glycolysis. The pmf largely consisted of a pH gradient of 1.3 units (Fig. 4A). Upon addition of 2 mM citrate, the internal pH did not increase further but additional pmf was generated as membrane potential (Fig. 4A, D). Addition of citrate to the cells alone resulted in a minor generation of a pH gradient without a change in the membrane potential

(Fig. 4B, E). Apparently, the flux through the pathway during the slow phase of citrate consumption (Fig. 2A) was too low to support a significant proton motive force. In contrast, the high flux observed in the presence of 0.2 mM L-lactate resulted in a significant increase in both ΔpH (0.6 pH units) and ΔΨ (Fig. 4C, F). Importantly, both the BCECF and $DiSC_3$ probes had an inhibitory effect on the rate of citrate consumption (not shown). In the absence of glucose or lactate, the second fast phase (Fig. 2B) was not apparent within 60 min and the energetics of the second phase could not be evaluated. Furthermore, the highest gradients obtained in the presence of L-lactate required a 10-fold higher concentration than required for the optimal rate of citrate consumption in the absence of the probes (Fig. 3A).

Figure 4. Energetics of citrate metabolism in *L. lactis* IL1403(pFL3).



The internal pH (A, B, C) and membrane potential (D, E, F) of the cells were continuously monitored in time. At the times indicated by the arrows, 0.5 mM glucose and 2 mM citrate (A, D), 2 mM citrate (B, E), and 2 mM citrate and 1 mM L-lactate (C, F) were added. Cells were loaded with BCECF as described in the Materials and methods section. Changes in the membrane potential were qualitatively evaluated from the quenching of the potentiometric probe $DiSC_3$.

Products of citrate metabolism

Citrate conversion by LAB to acetoin and acetate is redox neutral and no ATP is produced or

consumed (Fig. 1A) indicating that in resting cells the pathway is driven by the free energy gradient over the overall reaction and only counteracted by the built up of pmf. Nevertheless, the yield of the conversion was far from 100 %, indicating that other products formed preferably, especially when only citrate was present. Table 1 presents the products of citrate metabolism by resting cells of *L. lactis* IL1403(pFL3) for the three experimental conditions discussed above. In the presence of glucose when the highest yield of acetoin was obtained (67 %), two other products were found outside the cells, the precursor of acetoin α-acetolactate (22 %) and a small amount of acetate (11 %). Please note that this amount of acetate is in addition to the 2 mM formed in the citrate lyase reaction. Glucose was stoichiometrically converted to two molecules of L-lactate. In the presence of 0.2 mM L-lactate, the lower yield of acetoin (50 %) was mainly compensated for by an increased yield of α-acetolactate (36 %), while the additional acetate produced remained more or less the same (12 %). In the absence of any further additions, when no citrate was converted to acetoin, the product profile changed dramatically. Approximately equal fractions of the flux were directed to α-acetolactate and acetate (38 and 33 %, respectively), while in addition a significant amount of pyruvate (19 %) was produced. A minor amount of citrate was converted to oxaloacetate (6 %). It follows that in addition to a significant amount of acetate, intermediates of the citrate metabolic pathway to acetoin show up as products outside the cells in amounts that depended on the conditions. In all cases, the increase in time followed the decrease of the citrate concentration in time (not shown). Following full conversion of citrate, the slow increase in the acetoin concentration mentioned above in the presence of glucose and L-lactate (see Fig. 2A, C) was accompanied with a slow decrease of α-acetolactate indicating the reuptake of the latter by the cells and, subsequent, conversion into the former (not shown). Under all three conditions, an amount of acetate was formed ranging from ~10-40 % indicating that citrate metabolism in resting cells of IL1403(pFL3) diverts to two pathways yielding acetoin and acetate.

Exchange with metabolic intermediates

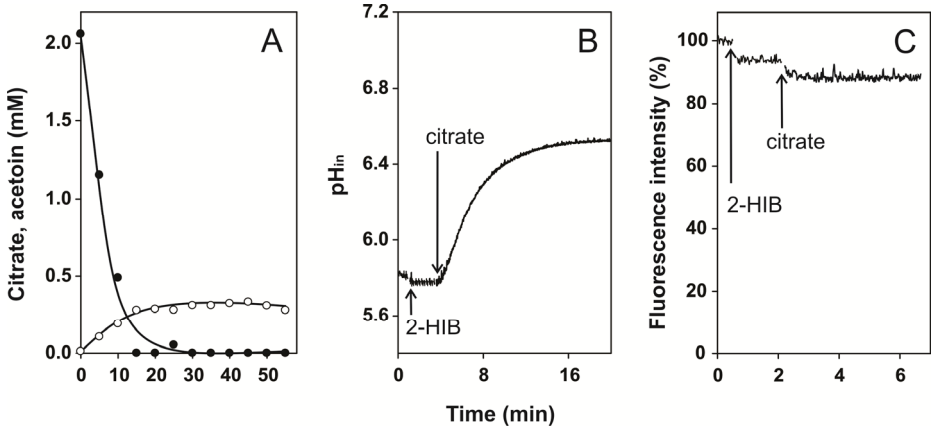
Excretion of pyruvate, α-acetolactate, and acetate during the fast second phase of citrate consumption by resting cells when only citrate was present (Table 1 and Fig. 2B) suggests that citrate was taken up in exchange with one or more of these metabolic products. The enhancement of the rate of citrate metabolism by L-lactate provides an assay for substrates of CitP (Fig. 2C). Any weak acid that is a substrate of CitP and membrane permeable should be able to induce rapid citrate consumption by *L. lactis* IL1403(pFL3) cells by the shuttle mechanism described for L-lactate in Fig. 1C. In agreement, the fast mode of citrate consumption was observed in the presence of the non-physiological substrate 2-hydroxyisobutyrate (2-HIB) (22) (Fig. 5A). A concentration of 2 mM 2-HIB was required to get the same rate as in the presence of 0.2 mM L-lactate. The amount of acetoin produced under this condition was considerably lower (0.32 mM *vs.* 0.5 mM) which was mainly compensated for by an increase in the production of α-

Table 1. Rates of citrate consumption and products formed by resting cells of *L. lactis* IL1403 (pFL3) from 2 mM of citrate in presence and absence of different substrates.

Condition[a]	Concn (mM)	*v*[b] (mM/min)	Products (mM)					Total (mM)[d]
			Oxaloacetate	Pyruvate	α-Acetolactate	Acetate[c]	Acetoin	
Glucose	0.1[e]	0.18	0	0	0.23 ± 0.03	0.23 ± 0.02	0.67 ± 0.05	2.03 ± 0.18
L-lactate	0.2	0.17	0	0	0.36 ± 0.01	0.25 ± 0.02	0.50 ± 0.01	1.97 ± 0.06
2-HIB	2	0.17	0	0	0.47 ± 0.08	0.40 ± 0.03	0.32 ± 0.01	1.91 ± 0.12
-	-	0.08[f]	0.13 ± 0.01	0.37 ± 0.03	0.38 ± 0.06	0.67 ± 0.10	0	1.86 ± 0.26
Acetate	2	0.046	0.05 ± 0.01	0.4 ± 0.01	0.39 ± 0.02	0.75 ± 0.06	0	1.98 ± 0.10
Pyruvate	2	0.087	0.08 ± 0.01	0	0.28 ± 0.08	1.4 ± 0.04	0	2.04 ± 0.13
α-Acetolactate/Acetate	2/2	0.06	0.09 ± 0.01	0.75 ± 0.08	0	1.2 ± 0.09	0	2.04 ± 0.18

[a] substrate present in addition to 2 mM citrate;
[b] cell OD_{660}=1.5; *v*, rate;
[c] in addition to acetate formed by citrate lyase;
[d] in equivalents of citrate (mM);
[e] converted to 0.2 mM of L-lactate;
[f] second phase (Fig. 2).

Figure 5. Citrate metabolism in *L. lactis* IL1403(pFL3) in the presence of 2-hydroxyisobutyrate (2-HIB).



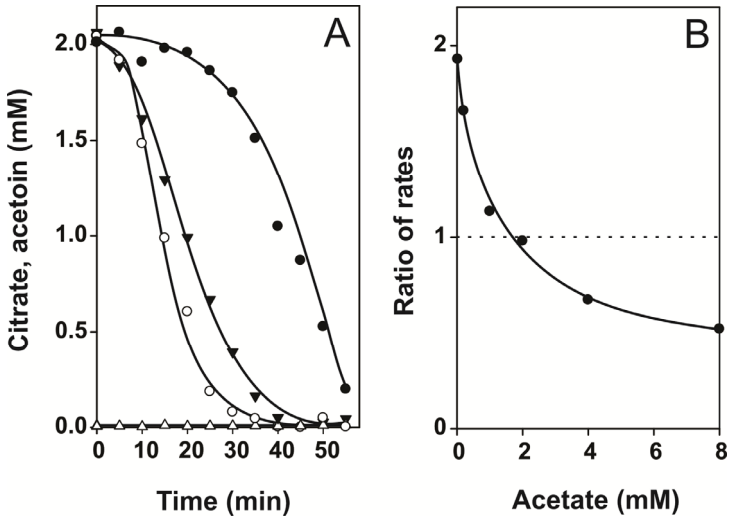
A. A concentration of 2 mM of citrate and 2 mM of 2-HIB was added to resting cells and the concentrations of citrate (●) and acetoin (○) were measured in the supernatants. B. The internal pH (B) and membrane potential (C) of the cells were monitored in time. 8 mM of 2-HIB and 2 mM citrate were added to the cells as indicated by the arrows.

acetolactate (Table 1). The energetics of the pathways in the presence of 2-HIB and L-lactate were the same as evidenced by the generation of ΔpH and ΔΨ, even though the magnitude of the latter was lower than observed in the presence of L-lactate (Fig. 5B, C).

The ability of the weak acids pyruvate, α-acetolactate, and acetate to function in citrate exchange catalyzed by CitP was measured by their ability to enhance citrate metabolism. At a concentration of 2 mM, pyruvate and acetate resulted in fast consumption of citrate by the resting cells of *L. lactis* IL1403(pFL3) (Fig. 6A). For both substrates, a short delay was observed before the onset of the fast phase. The rates were slower than observed in the presence of glucose, L-lactate or 2-HIB, but in the same order of magnitude as observed in the second, fast phase in the presence of citrate alone (Table 1). The same experiment with α-acetolactate was complicated by the fact that the compound is not stable and is only commercially available as a diester. The release of α-acetolactate from the diester by hydrolysis results in equivalent amounts of acetate and ethanol (see Materials and methods). The presence of 2 mM of ethanol did not have a significant effect on the citrate consumption rate (not shown). At concentrations lower than 2 mM, the equimolar mixture of α-acetolactate and acetate (and ethanol) induced a higher rate of citrate consumption than observed in the presence of acetate alone demonstrating exchange of citrate and α-acetolactate (Fig. 6B). At higher concentrations, α-acetolactate in the mixture inhibited the rate observed with acetate alone, the same phenomenon as observed for L-lactate (Fig. 3A).

The results suggest that uptake of citrate by CitP competes for exchange with the intermediates pyruvate and α-acetolactate and the end products L-lactate and acetate. In agreement, increasing the concentration of L-lactate added to the cells in the presence of citrate, resulted in a decrease of the appearance of α-acetolactate outside the cells (Fig. 3B). Apparently, more citrate was taken

Figure 6. Citrate uptake in exchange with metabolic intermediates in *L. lactis* IL1403(pFL3).

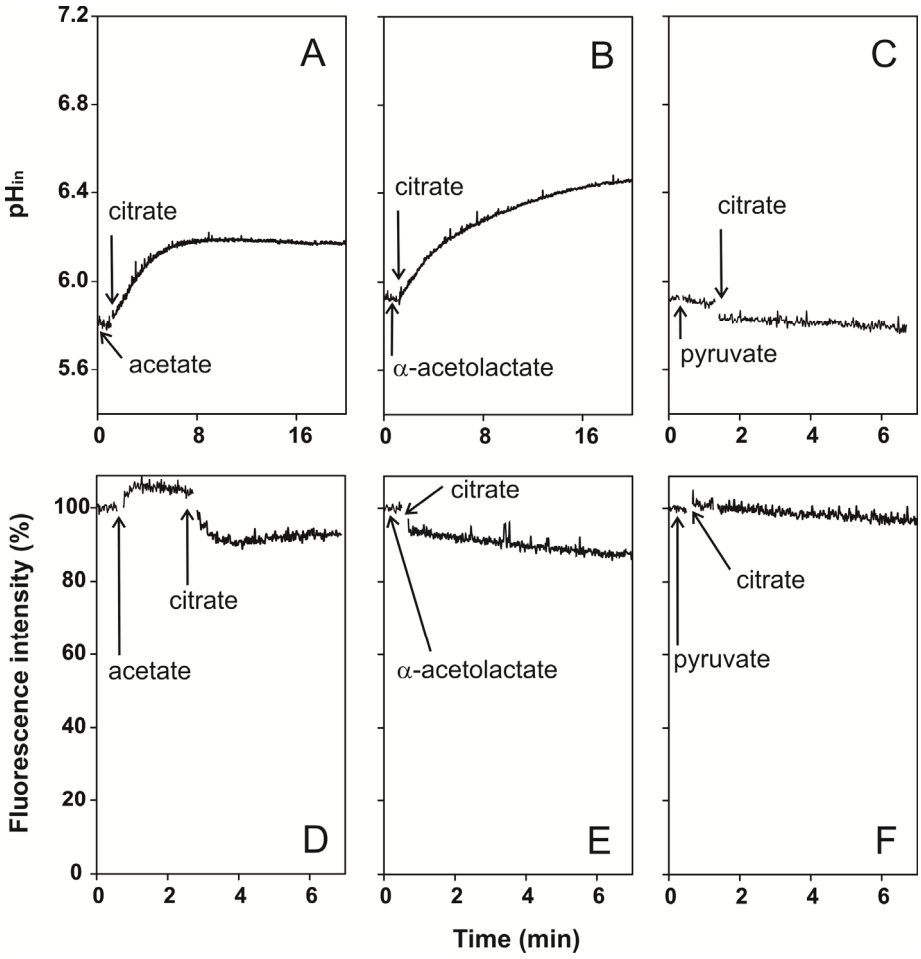


A. A concentration of 2 mM of citrate was added to resting cells in the presence of 2 mM pyruvate (○), 2 mM acetate (▼) and no further additions (●). The citrate (○, ▼, ●) and acetoin (Δ) concentrations in the supernatant were measured at the indicated time points. B. Ratio of the rates of citrate consumption when 2 mM of citrate was added to the cells in the presence of α-acetolactate freshly prepared from the diester (see Materials and methods) and acetate in the concentration range of 20 μM to 8 mM of the latter. Freshly prepared α-acetolactate contains an equivalent concentration of acetate. Rates were deduced from the decrease in citrate concentrations during the second phase assuming zero-order kinetics.

up in exchange with L-lactate added to the cells and less with α-acetolactate produced by the cells, which, consequently, resulted in a higher production of acetoin. When L-lactate becomes inhibiting the yield of acetoin goes down again.

In line with the lack of acetoin production in the presence of citrate alone, neither acetate, nor pyruvate or α-acetolactate induced the production of acetoin (Table 1). The product profile in the presence of acetate was very similar to the profile observed with citrate alone; the flux was evenly distributed over the acetoin and acetate pathways and a significant amount of pyruvate was excreted from the cells. Under these conditions, a pH gradient was generated of 0.4 units and, in addition, membrane potential was generated (Fig. 7A, D). In the presence of pyruvate and α-acetolactate (plus acetate) the product profile changed considerably. Both prevented the production of additional amounts of the added compound and shifted the flux to acetate production. In the presence of 2 mM α-acetolactate plus 2 mM acetate, the pathway to α-acetolactate and acetoin was completely absent and only pyruvate and acetate was produced. Then, a pH gradient of 0.5 units was generated without an effect on the membrane potential (Fig. 7B, E). Remarkably, citrate metabolism in the presence of pyruvate did not result in the generation of a detectable proton motive force (Fig. 7C, F).

Figure 7. Energetics of citrate metabolism in *L. lactis* IL1403(pFL3) in the presence of metabolic intermediates.



The internal pH (A, B, C) and membrane potential (D, E, F) of the cells were continuously monitored in time. At the times indicated by the arrows, 2 mM acetate and 2 mM citrate (A, D), 2 mM α-acetolactate (plus acetate) and 2 mM citrate (B, E), and 8 mM pyruvate and 2 mM citrate (C, F). Cells were loaded with BCECF as described in the Materials and methods section. Changes in the membrane potential were qualitatively evaluated from the quenching of the potentiometric probe $DiSC_3$.

Discussion

Metabolic state of the cells

In this study, citrate metabolism was studied in the presence and absence of other substrates in resting cells of *L. lactis* IL1403(pFL3). The cells were grown in batch culture in rich medium (M17) at an initial pH of 7 and supplemented with glucose as energy and carbon source. The cells were harvested at a relatively low density (OD_{600} of 0.6) when the pH had dropped to 6.8. Under these conditions, *L. lactis* ferments glucose by a homofermentative metabolism producing L-lactate (14, 42). In the experiments, citrate metabolism is catalyzed by the complement of enzymes present in

the cells under those growth conditions. The metabolic state is typical for exponential growth when energy is not limiting and not typical for citrate fermentation conditions.

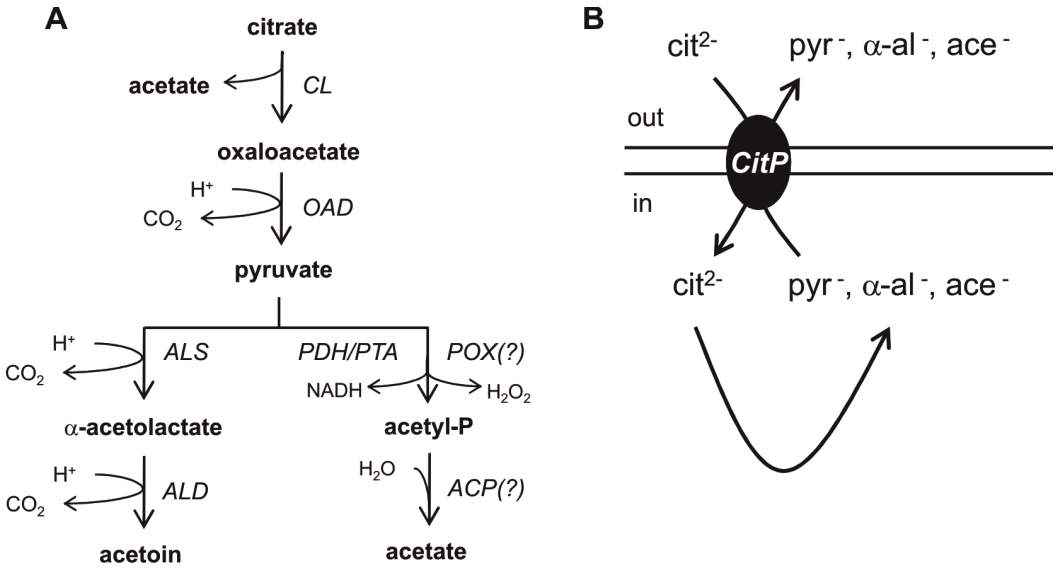
In citrate fermenting *L. lactis* strains, the citrate transporter CitP is encoded on an endogenous plasmid (24), while the metabolic enzymes are encoded on the chromosome (8). Expression of the *citP* gene is induced by the presence of citrate under acidic conditions in line with the physiological function of the citrate fermentation pathway in acid stress resistance in *L. lactis* CRL264 (15, 24). Strain IL1403 (10) is a plasmid free strain and, therefore, a citrate permease mutant of the biovar *diacetylactis*. Transfer of plasmid pFL3 to IL1403 yielding IL1403(pFL3), converted to the strain the ability to produce acetoin and diacetyl (9). Plasmid pFL3 contains the lactococcal *citP* gene under control of the *S. pneumonia polA* promoter (24). Expression of the neither transporter nor plasmid copy number is controlled by citrate or the pH of the medium. In agreement, resting cells of *L. lactis* IL1403(pFL3) grown in the media with an initial pH of 6 or 7, or in the presence or absence of 10 mM citrate showed no significant difference in citrate consumption rate (not shown).

Broadening of CitP substrate specificity

The citrate transporter CitP plays a pivotal role in the energetics of the citrate fermentation pathway in LAB by catalyzing electrogenic exchange of divalent citrate and monovalent lactate. It was shown before that CitP has broad substrate specificity, translocating a wide range of physiological and non-physiological 2-hydroxycarboxylates (4, 6). The present study demonstrates an even broader specificity of CitP. The intermediates α-acetolactate and pyruvate and the end product acetate were shown to be substrates of CitP (Fig. 8B). α-Acetolactate was not a surprise because it contains the 2-hydroxycarboxylate motif. Also, some activity with the 2-ketoacid pyruvate was already observed in studies with membrane vesicles (4). Possibly, the hydrated form of pyruvate (2,2-dihydroxypropionate) is the real substrate. Exchange activity with acetate was not observed before. It may be noted that CitW of *K. pneumoniae*, another member of the 2-HCT family catalyzes citrate/acetate exchange and is believed to function in citrate fermentation in this Gram-negative bacterium (20). The broad specificity of CitP for physiological substrates has implications for the citrate metabolic pathway in resting cells. A significant flux through the pathway requires CitP to operate in the exchange mode. Depending on their availability and concentration inside and outside the cell, CitP uses pyruvate, α-acetolactate, acetate, or L-lactate in the exchange mode to take up citrate from the medium. In their absence, citrate is taken up by CitP operating in the slow symport mode (5) and an internal pool of intermediates and the product acetate has to built up before CitP can switch to the fast exchange mode which results in a lag time observed in the consumption of citrate (Fig. 2B). Then, metabolic intermediates pyruvate and α-acetolactate are produced as the products of the pathway. In their presence, CitP operates in the exchange mode from the beginning via the shuttle mechanism depicted in Figure 1C. The product profile depends on the competition between the different

substrates of CitP present in the system.

Figure 8. Citrate metabolism in *L. lactis* IL1403(pFL3).



A. Pathway for the conversion of citrate to acetoin and to acetate. Enzymes: CL, citrate lyase; OAD, oxaloacetate decarboxylase; ALS, α-acetolactate synthase; ALD, α-acetolactate decarboxylase; POX, pyruvate oxidase; PDH, pyruvate dehydrogenase complex; PTA, phosphotransacetylase; ACP, acylphosphate phosphohydrolase. The stoichiometry of the reactions was not taken into account. B. Exchange of divalent citrate (cit^{2-}) and citrate metabolic products: pyruvate (pyr^{-}), α-acetolactate ($α\text{-}al^{-}$), and acetate (ace^{-}) catalyzed by CitP.

Diverse fates of citrate

Analysis of the products formed from citrate under various conditions by resting cells of *L. lactis* IL1403(pFL3) revealed the metabolic pathway depicted in Fig. 8A. Citrate is converted to pyruvate followed by branching of the pathway yielding either acetoin or acetate. The profile of product formation under various conditions can be divided into two main groups (see Table 1). In one group, citrate is taken up by CitP in exchange with a substrate that is not formed from citrate, while in the other group it is. L-lactate, produced from glucose or added to the buffer, and 2-HIB are not part of citrate metabolism and their involvement is restricted to the transport step. With these substrates the rate of citrate consumption was highest and 80-90 % of pyruvate produced was directed to the acetoin route. Still a significant fraction did not make it to acetoin and was excreted as α-acetolactate indicating that this 2-hydroxycarboxylate competes successfully with L-lactate and 2-HIB in the exchange reaction catalyzed by CitP. In the other group, the flux distribution is mainly determined by the availability of exchangeable substrates. The overall rate of citrate consumption was lower and the fraction of pyruvate directed to the acetoin route dropped to 0-40 %. All α-acetolactate produced was used to take up citrate in the exchange process and,

consequently, no acetoin was produced under these conditions. In addition, a significant fraction of pyruvate up to 40 % was spent to take up citrate and ended up outside the cells. The remainder of the flux was directed to the acetate route which amounted to 60-70 % in the presence of the intermediates pyruvate and α-acetolactate.

Energetics of citrate metabolism

Citrate metabolism in LAB yielding acetoin results in the generation of pmf by a secondary metabolism that involves membrane potential generation by electrogenic citrate/lactate exchange and pH gradient generation by proton consumption in the decarboxylation reactions in the pathway. The two contributions are indirectly coupled since the specificity of CitP determines the net number of protons that enter the cell in the transport step (Fig. 1). Citrate metabolism in resting cells of *L. lactis* IL1403(pFL3) generated the highest pmf in the presence of L-lactate and 2-HIB, conditions characterized by the highest flux through the pathway and highest yield of acetoin (see above). These conditions reflect the situation in growing cells during citrate/glucose cometabolism. Under conditions when citrate was taken up in exchange with the intermediates pyruvate and α-acetolactate and the end product acetate, when the flux was lower and the routing more diverse, the generated pmf was lower or even absent. The pmf generated will be the sum of the contributions of the fluxes to external pyruvate, α-acetolactate, and acetate, suggesting that not all routes are equally effective in pmf generation. In particular, in the presence of pyruvate when the highest yield of acetate was observed, no pmf was detected (Fig. 7C) strongly suggesting that the acetate route does not generate metabolic energy.

Pathway to acetate

The lack of pmf generation in the pathway from pyruvate to acetate is surprising since normally the conversion proceeds via acetate kinase and yields ATP. Based on the enzyme complement encoded in the genome of *L. lactis* IL1403 (8) three different routes result in the conversion of pyruvate to acetate, all three proceeding through acetyl-P. Acetyl-P may be formed from acetyl-CoA produced from pyruvate by pyruvate formate lyase (PFL) or pyruvate dehydrogenase (PDH), and directly from pyruvate by pyruvate oxidase (POX). PFL produces in addition formate which was not detected as a product ruling out the involvement of PFL which is known to act only under strict anaerobic conditions (1). PDH produces in addition NADH which has to be reoxidized to allow turnover through the route (39). *L. lactis* is an aerotolerant lactic acid bacterium that contains NADH oxidases (encoded by the *noxC*, *noxD*, *noxE* genes) (8) that protect the cell during aerobic growth (11). NOX enzymes catalyze oxidation of NADH to NAD^+ by molecular oxygen and are induced under aerobic conditions (7, 23, 38). The combination of PDH and NOX enzymes could account for the conversion of pyruvate to acetyl-CoA under the conditions of the experiment. Acetyl-CoA produced by PDH is normally converted to acetaldehyde and ethanol, both of which were not detected in the supernatants which leaves only the conversion to acetyl-P

catalyzed by phosphotransacetylase (PTA). The third enzyme, POX (encoded by the *poxL* gene) produces in addition to acetyl-P hydrogen peroxide. Low amounts of H_2O_2 amounting to a few percent of the acetate produced were detected in the supernatants (not shown), suggesting that the enzyme does not play an important role, if any. However, an intriguing possibility might be that H_2O_2 produced by POX is used to reoxidize NADH produced by PDH. The enzyme catalyzing the reaction is alkylhydroperoxide reductase (gene name *ahpC*) which plays a role in detoxification (8, 19). Acetyl-P is converted by acetate kinase to acetate. However, the acetate kinase reaction is coupled to the production of ATP which should result in the generation of pmf. ATP produced from as little as 0.1 mM of glucose generated a pH gradient of 1.3 units (Fig. 4A). Citrate metabolism yielding 1.4 mM of acetate did not generate pmf (Table 1 and Fig. 7C) strongly suggesting that no ATP is formed and that acetate kinase is not involved. Possibly, the enzyme encoded by the *yfjC* gene (8) which is homologous to acylphosphate phosphohydrolases that catalyze hydrolysis of acetyl-P plays a role. The details of the pathway from pyruvate to acetate identified here still have to be resolved.

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Chapter 3

Mechanism of citrate metabolism by an oxaloacetate decarboxylase deficient mutant of *Lactococcus lactis* IL1403

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3

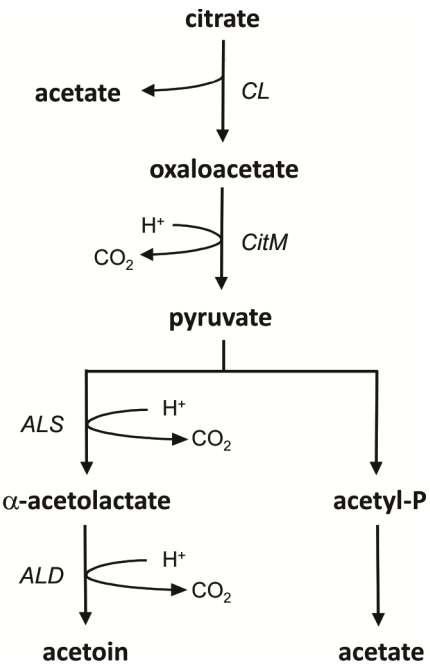
Abstract

Citrate metabolism in resting cells of *Lactococcus lactis* IL1403(pFL3) results in the formation of two end products from the intermediate pyruvate, acetoin and acetate (Pudlik, A. M. and Lolkema, J. S. 2011. *J. Bacteriol.* 193:706-714.). Pyruvate is formed from citrate following uptake by the transporter CitP through the subsequent actions of citrate lyase and oxaloacetate decarboxylase. The present study describes the metabolic response of *L. lactis* when oxaloacetate accumulates in the cytoplasm. The oxaloacetate decarboxylase deficient mutant ILCitM(pFL3) showed nearly identical rates of citrate consumption, but the end product profile in the presence of glucose shifted from mainly acetoin to only acetate. In addition, in contrast to the parental strain, the mutant strain did not generate proton motive force. Citrate consumption by the mutant strain was coupled to the excretion of oxaloacetate with a yield of 80-85 %. Following citrate consumption, oxaloacetate was slowly taken up by the cells, converted to pyruvate by a cryptic decarboxylase and, subsequently, to acetate. Transport of oxaloacetate is catalyzed by CitP. The parental strain IL1403(pFL3) containing CitP, consumed oxaloacetate, while the original strain IL1403, not containing CitP, did not. Moreover, oxaloacetate consumption was enhanced in the presence of L -lactate indicating exchange between oxaloacetate and L-lactate catalyzed by CitP. Hence, when oxaloacetate inadvertently accumulates in the cytoplasm, the physiological response of *L. lactis* is to excrete oxaloacetate in exchange with citrate by an electroneutral mechanism catalyzed by CitP. Subsequently, in a second step, oxaloacetate is taken up by CitP and metabolized to pyruvate and acetate.

Introduction

Citrate metabolism plays an important role in many food fermentation processes involving Lactic Acid Bacteria (LAB) (11). Citrate is the precursor of carbon dioxide and the flavor compounds diacetyl and acetoin that contribute to the organoleptic properties of fermented foods. The flavor compounds are formed from the central metabolite pyruvate in the cytoplasm. Citrate metabolism feeds directly into the pyruvate pool (Fig. 1). Following uptake by the secondary citrate transporter CitP, citrate lyase converts citrate to acetate and oxaloacetate. Acetate is not further metabolized and leaves the cells, while oxaloacetate is decarboxylated to pyruvate by a soluble oxaloacetate decarboxylase encoded by the *mae* gene and termed CitM (25). In a recent study it was demonstrated that in the lactic acid bacterium *Lactococcus lactis* IL1403(pFL3) part of pyruvate formed from citrate was converted into acetoin and part into acetate in parallel pathways (23). In resting cells, the distribution over the two end products strongly depended on the conditions. A high turnover rate through the pathway favored the formation of acetoin over acetate.

Figure 1. Schematic representation of citrate metabolism by *L. lactis* IL1403 (pFL3).



Enzymes: CL, citrate lyase; CitM, oxaloacetate decarboxylase; ALS, α-acetolactate synthase; ALD, α-acetolactate decarboxylase. The stoichiometry of the reactions was not taken into account.

The citrate transporter CitP plays a pivotal role in the kinetics of the pathway. Under physiological conditions, when citrate is cometabolized with a carbohydrate, CitP operates in the fast citrate/L-lactate exchange mode; citrate is taken up and at the same time L-lactate, the product of glycolysis, is excreted (precursor/product exchange) (2, 17, 19, 23). In the absence of L-lactate, CitP operates in the much slower unidirectional H^+-symport mode (19), which makes uptake the rate determining step for the flux through the pathway (23). It was shown that, in addition to L-lactate, CitP has affinity for intermediates/products of the pathway, which results in the typical biphasic consumption of citrate in the absence of L-lactate. During the first phase, citrate is taken up slowly in symport with a H^+, but as intermediates/products accumulate in the cytoplasm, the transporter switches to the fast exchange mode in which citrate is taken up in exchange with pyruvate, α-acetolactate, and (or) acetate, resulting in a much faster consumption rates in the second phase. Therefore, the kinetics and product profile of citrate metabolism is largely determined by the availability of exchangeable substrates for the transporter CitP.

The physiological function of citrate metabolism in LAB is in metabolic energy generation and acid

stress resistance (8, 18, 20, 28). The citrate metabolic pathway generates proton motive force (pmf) (3, 19, 20) by an indirect proton pumping mechanism, by which the two components of the pmf, membrane potential (Δψ) and pH gradient (ΔpH), are generated in separate steps (13, 14). Exchange of divalent citrate and monovalent lactate catalyzed by CitP generates membrane potential of physiological polarity (i.e. inside negative). In *L. lactis* IL1403(pFL3), exchange of citrate and the intermediates/products acetate and α-acetolactate was shown to be electrogenic as well (23). Proton consumption in the decarboxylation of oxaloacetate and α-acetolactate, as well as the formation of the latter (see Fig. 1), result in a pH gradient (inside alkaline). The pathway from pyruvate to acetate, which has not been resolved completely, does not seem to generate pmf (23). The pmf may be used to produce additional ATP via F_1F_0-ATPase or to maintain a high cytoplasmic pH to improve acid resistance of the strain.

A recent study reported on the consequences of a null allele of oxaloacetate decarboxylase in strain *L. lactis* ILCitM(pFL3) on growth and intracellular pH maintenance in the presence and absence of citrate (1). While no effect on the maintenance of the cytoplasmic pH was observed, growth was impaired in the presence of citrate, which was correlated with the transient accumulation of oxaloacetate in the medium. Transporters for oxaloacetate are rarely found in bacteria. The dicarboxylate transporters DccT of *Corynebacterium glutamicum* (31), SdcL of *Bacillus licheniformis* (29), as well as the DctA transporters of *C. glutamicum* (32) and *B. subtilis* (10) were shown to be competitively inhibited by oxaloacetate. In the present study, the physiological response of the oxaloacetate deficient mutant to potentially toxic accumulation of oxaloacetate in the cytoplasm is demonstrated. Mechanistic basis of citrate metabolism in the mutant is the ability of the transporter CitP to excrete oxaloacetate in exchange with citrate and to take up oxaloacetate in exchange with L-lactate or another intermediate/product of the pathway.

Materials and methods

Bacterial strains and growth condition

Lactococcus lactis IL1403 (6), IL1403(pFL3) (16), and an oxaloacetate decarboxylase mutant of IL1403(pFL3) named ILCitM(pFL3) (1) were used in this study. Plasmid pFL3 harbours the *citP* gene under control of the *Streptococcus pneumoniae polA* promoter (16). Expression nor plasmid copy number are under control of citrate or pH in strain IL1403(pFL3) (8). Mutant strain ILCitM was constructed from IL1403 by a deletion of 14 bp between position 584 and 598 of the oxaloacetate decarboxylase gene encoded by the *mae* (1). Precultures were grown overnight at 30 ^{o}C in M17 broth medium supplemented with 0.5 % (w/v) glucose (M17G) and 5 μg ml^{-1} of tetracycline when appropriate. Cells were grown in M17G medium with an initial pH adjusted to 7.0. Growth was performed in 100 ml serum bottles without agitation and at 30 ^{o}C. Growth was followed by measuring the optical density at a wavelength of 660 nm. Cells were harvested at mid -exponential growth phase when the optical density was 0.6 by spinning for 10 min at 3000 rpm.

Cells were washed two times with 50 mM potassium phosphate pH 5.8 buffer, and finally, resuspended in the same buffer of 4 ^{o}C.

Citrate and oxaloacetate consumption by resting cells

Resting cells at an OD_{660} of 1.5 in 50 mM potassium phosphate pH 5.8 buffer were incubated at 30 ^{o}C without agitation for 10 min. The assay was performed in a total volume of 1.5 ml. At t=0, citrate or oxaloacetate was added at a concentration of 2 mM. When indicated, 0.1 mM of glucose or 0.2 mM of L-lactate was added together with citrate. Samples of 100 µl were taken every 5 or 10 min and immediately centrifuged for 0.5 min at maximum speed in a table top centrifuge. The supernatant was stored on ice until further analysis by enzymatic assays and/or HPLC. Measurements of the concentrations of citrate, oxaloacetate, and pyruvate were in good agreement between the two methods. Each experiment was done at least in triplicate and the averages and standard deviation of three independent experiments were shown.

Enzymatic assays

Citrate, oxaloacetate, and pyruvate were measured as described before (23), using the commercially available enzymes citrate lyase (CL), L-malate dehydrogenase (L-MDH), and L-lactate dehydrogenase (L-LDH). Briefly, an aliquot of 30 µl of the sample was added to 50 mM glycyl-glycine pH 7.8 buffer containing NADH and L-MDH. Oxaloacetate in the sample is converted to L-malate at the expense of NADH. Subsequently, pyruvate in the same sample was measured by addition of L-LDH which results in the conversion of pyruvate to L-lactate at the expense of NADH. Subsequent addition of CL converts citrate in the sample to oxaloacetate (and pyruvate) resulting in an additional decrease in the NADH concentration equivalent to the citrate concentration present in the samples. The assay was performed in 96-well microtiter plates. The decrease in NADH concentration was measured spectroscopically at 340 nm.

HPLC analysis

Products of citrate metabolism were determined by loading an aliquot of 10 µl of the supernatant on an Aminex HPX-87H anion-exchange column (Bio-Rad Laboratories, Inc., Richmond, CA) operated at 30 ^{o}C in isocratic mode using 0.005 M H_2SO_4 as the mobile phase and a flow rate of 0.8 ml/min. Quantification was based on calibration curves generated by injecting standards of known concentrations.

Measurement of internal pH (ΔpH) and membrane potential (ΔΨ)

The components of the proton motive force were measured as described before (23). To measure ΔpH, resting cells resuspended to high density (typically containing 50 mg/ml of protein) in 50 mM potassium phosphate pH 5.8 buffer were loaded with 2',7'-bis-(2-carboxyethyl)-5(and-6)-carboxyfluorescein (BCECF). Fluorescence measurements were performed in 1 cm cuvettes

containing 50 mM potassium phosphate pH 5.8 buffer equilibrated at 30 ^{o}C and cells loaded with BCECF. The cuvette was stirred with a magnetic stirring bar. Fluorescence was measured using excitation and emission wavelengths of 502 and 525 nm, respectively, with slit widths of 4 and 16 nm, respectively. The fluorescence signal was sampled every second. Opening of the measurement compartment caused loss of data during the first 5-6 seconds after an addition to the cuvette was made. The cytoplasmic pH was calculated as described by (21).

Membrane potential was measured qualitatively with the fluorescent probe 3,3'-dipropylthiocarbocyanine iodide ($DiSC_3$) (26). Decrease in fluorescence intensity correlates with an increase in electrical potential across the membrane. $DiSC_3$ was added from a stock solution to a final concentration of 2 µM to the quartz cuvettes containing with 50 mM potassium phosphate pH 5.8 buffer and cells. The system was left to equilibrate for 5 min at 30 ^{o}C. Fluorescence measurements were performed using excitation and emission wavelengths of 500 and 705 nm, respectively, and slit widths of 8 nm, respectively.

Chemicals

L-lactate dehydrogenase (L-LDH), L-malate dehydrogenase (L-MDH), and citrate lyase (CL) were obtained from Roche Applied Science. 2',7'-bis-(2-carboxyethyl)-5(and-6)-carboxyfluorescein (BCECF, acid form) and 3,3'-dipropylthiocarbocyanine iodide ($DiSC_3$) probes were obtained from Invitrogen Molecular Probes.

Results

Citrate metabolism by L. lactis ILCitM(pFL3)

The consumption of citrate and the formation of the end products acetoin and acetate were studied in resting cells of *L. lactis* strains IL1403(pFL3) and ILCitM(pFL3) in the presence and absence of glucose or L-lactate. Both strains express the citrate transporter CitP in trans from a non-native promoter to decouple *citP* expression from the native control (16). CitP encoded on plasmid pFL3 completes together with the metabolic enzymes encoded on the chromosome (5) the citrate metabolic pathway. Strains ILCitM(pFL3) contains a frame shift deletion of 14 base pairs in the *mae* gene resulting in a null allele of the oxaloacetate decarboxylase CitM (Fig. 1) (1). Both strains were grown until the mid-exponential growth phase (OD_{660}=0.6) in M17 broth medium at pH 7.0 supplemented with 0.5 % (w/v) glucose and in the absence of citrate (23).

Resting cells of strain IL1403(pFL3) resuspended at an OD_{660} of 1.5 in 50 mM KP_i pH 5.8 buffer rapidly consumed 2 mM of citrate in the presence of 0.1 mM of glucose (Fig. 2A). Surprisingly, the oxaloacetate deficient strain ILCitM(pFL3) which is blocked in the citrate metabolic pathway (see Fig. 1) showed more or less the same consumption rate. The rates were 0.17 and 0.16 mM/min for the parental and mutant strains, respectively (Table 1). Under this condition in the parental strain, citrate uptake is catalyzed by the transporter CitP in exchange with L-lactate produced

Table 1. Rates of citrate consumption (*v*) in *L. lactis* IL1403(pFL3) and ILCitM(pFL3) in the presence and absence of glucose and L-lactate.

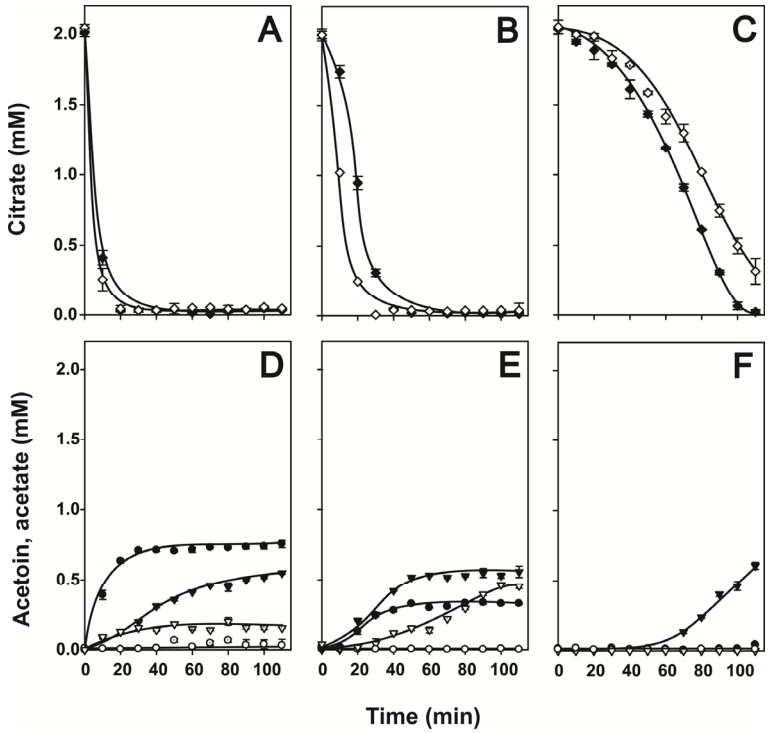
Addition[a]	*v* (mM/min)	
	IL1403(pFL3)	ILCitM(pFL3)
0.1 mM glucose	0.17 ± 0.02	0.16 ± 0.01
0.2 mM L-lactate	0.09 ± 0.01	0.07 ± 0.01
none	0.025 ± 0.003[b]	0.029 ± 0.003[b]

[a] in addition to 2 mM citrate;
[b] second phase (Fig. 1).

from glucose. The conditions may be mimicked by adding L-lactate rather than glucose directly to the buffer (Fig. 2B). L-lactate, present at a substoichiometric concentration relative to citrate (0.2 and 2 mM, respectively), allows CitP to operate in the fast exchange mode via the so-called 'shuttle' mechanism. L-lactate exported from the cells by CitP re-enters as L-lactic acid by passive diffusion (19, 23). Similarly as observed in the presence of glucose, the rate of citrate consumption in the presence of L-lactate was only marginally slower in the oxaloacetate deficient mutant as compared to the parental strain with rates of 0.07 and 0.09 mM/min, respectively. A short delay was observed for the mutant strain that was not observed with the parental strain (Fig. 2B). In the absence of L-lactate (or glucose) citrate consumption by the parental strain is characterized by two distinct phases (Fig. 2C) (23). During the first slow phase, intermediates and products of the pathway built up in the cytoplasm, which in the second fast phase are excreted in exchange for citrate. The mutant strain showed the same behavior, but the first phase was shorter and the rate in the second phase higher (Fig. 2C).

Metabolism of citrate in resting cells of *L. lactis* IL1403(pFL3) was shown to result in the production of two end products, acetoin and acetate, depending on the conditions (23). Acetoin was the major product of citrate metabolism when the rate of citrate consumption was fast, i.e. in the presence of glucose or L-lactate (0.67 and 0.5 mM, respectively) (Fig. 2D, E). A fast rate of citrate consumption results in accumulation of pyruvate in the cytoplasm which favors the acetoin synthesis pathway because of the low affinity of α-acetolactate synthase (ALS) for pyruvate (K_m=50 mM) (see Fig. 1) (27). The kinetics of acetoin formation (one molecule of acetoin is formed from two molecules of citrate) follows the kinetics of citrate degradation. In contrast, under the same conditions and with similar rates of citrate consumption, the mutant strain ILCitM(pFL3) did not produce any measurable acetoin, which would be in agreement with the pathway being interrupted following oxaloacetate. Instead, a significant amount of acetate, in addition to acetate produced by citrate lyase, was produced, suggesting, in contradiction, that the pathway following oxaloacetate was still functional. Acetate production by the mutant was clearly delayed in time. When all citrate was depleted, the amounts of additional acetate produced were 0.16 mM and 0.12 mM in the presence of glucose and L-lactate, respectively (Table 2). Following the depletion of citrate, the amounts of acetate increased to 0.55 mM and 0.42 mM, respectively (Table 2 and Fig. 2D, E). In the absence of glucose or L-lactate, the parental strain produced acetate in the second phase, but no acetoin (23) while neither of the products were detected in case of the

Figure 2. Citrate consumption and formation of the end products of citrate metabolism in *L. lactis* IL1403(pFL3) and ILCitM(pFL3).



Citrate consumption (◊, ♦) and acetoin (○, ●) and acetate (∇, ▼) formation by resting cells of *L. lactis* IL1403 (pFL3) (open symbols) and ILCitM(pFL3) (closed symbols) in the presence of 0.1 mM glucose (A, D), 0.2 mM L-lactate (B, E), and no further additions (C, F). At t=0, a concentration of 2 mM of citrate was added to the cell suspensions in 50 mM potassium phosphate pH 5.8 buffer.

mutant strain during the course of the experiment (Fig. 2F). The experiments demonstrate that the oxaloacetate deficient mutant metabolized citrate at similar rates as the parental strain but the product profile was significantly different. While the pathway to acetoin appeared to be blocked, the route to acetate appeared to be functional (Fig. 2).

Energetics of the citrate metabolic pathway in L. lactis ILCitM(pFL3)

Citrate metabolism in LAB generates metabolic energy by generating pmf. Membrane potential is generated in the citrate uptake step by electrogenic exchange of divalent citrate and monovalent lactate, while proton consumption in the decarboxylation reactions in the pathway result in a transmembrane pH gradient that is alkaline inside (18, 19, 20). The transmembrane pH gradient was evaluated by measurement of the internal pH inferred from the fluorescent dye BCECF that was trapped inside the cells (21). The membrane potential was measured qualitatively by the potentiometric probe $DiSC_3$ (26). Previously it was shown that both probes had an inhibitory effect on the flux through the citrate pathway in strain IL1403(pFL3), which in part could be compensated for by increasing the L-lactate concentration. Addition of 2 mM citrate in the

Table 2. Product formation from citrate by resting cells of *L. lactis* ILCitM(pFL3) under different conditions measured by HPLC.

Condition[a]	Mean concn of product (mM) ± SD				Mean yield[c] ± SD
	Oxaloacetate	Pyruvate	Acetate[b]	α-Acetolactate	
0.1 mM glucose					
20 min[d]	1.6 ± 0.01	0.17 ± 0.04	0.16 ± 0.05	0.03 ± 0.01	1.96 ± 0.11
110 min	0.82 ± 0.02	0.44 ± 0.08	0.55 ± 0.06	0.13 ± 0.02	1.94 ± 0.18
0.2 mM L-lactate					
40 min[d]	1.71 ± 0.02	0.14 ± 0.02	0.12 ± 0.09	0.02 ± 0.01	1.99 ± 0.14
110 min	1.2 ± 0.02	0.36 ± 0.04	0.42 ± 0.08	0.02 ± 0.02	2 ± 0.16
No addition					
100 min[d]	1.59 ± 0.01	0.33 ± 0.06	0	0.06 ± 0.01	1.98 ± 0.08

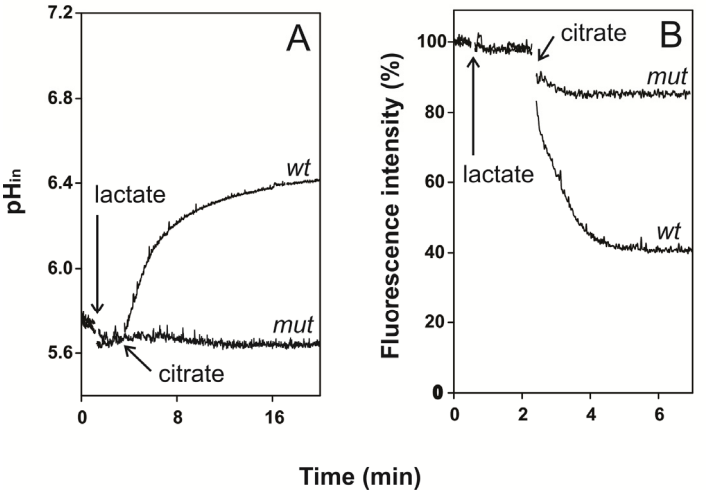
[a] in addition to 2 mM citrate; time points are shown in Fig. 4;
[b] in addition to the equimolar concentration of acetate formed by citrate lyase;
[c] in equivalents of citrate (2 mM);
[d] citrate depleted.

presence of 1 mM L-lactate resulted in a pH gradient ΔpH of 0.7 units that developed during the first 10 minutes of citrate consumption (Fig. 3A). The kinetics of membrane potential ΔΨ generation was faster. A steady state was reached within 2 minutes (Fig. 3B). The similar rate of citrate consumption by the mutant strain ILCitM(pFL3) (Fig. 2B) did not result in generation of ΔpH and only a small change in ΔΨ was observed (Fig. 3A, B). It should be noted that the measurements reflect steady state values of pmf obtained at a constant rate of citrate consumption (see Fig. 2B). For technical reasons, the methods do not allow for measurements over longer periods of time after all citrate is consumed. It follows that at the same high rate of citrate consumption, proton motive force generation is severely inhibited in oxaloacetate decarboxylase deficient mutant.

Oxaloacetate is excreted during citrate consumption in L. lactis ILCitM(pFL3)

Citrate metabolism in *L. lactis* IL1403(pFL3) resulted, in addition to the end products, in the appearance of citrate pathway intermediates, i.e. α-acetolactate and/or pyruvate, outside the cells. Oxaloacetate, the first intermediate of citrate breakdown, was found only in trace amounts and only when citrate was metabolized in the absence of glucose or L-lactate (23). In contrast, citrate metabolism in the mutant strain resulted in the production of a high amount of oxaloacetate outside the cells. In the presence of 0.1 mM glucose, 0.2 mM L-lactate, or no further additions, 2 mM of citrate resulted in 1.6, 1.71 and 1.59 mM of oxaloacetate outside the cells, respectively (Fig. 4 and Table 2). The increase of oxaloacetate was kinetically coupled to the decrease of citrate. Small amounts of pyruvate and/or acetate completed the carbon balance (Table 2).

Figure 3. Energetics of the citrate metabolism in *L. lactis* IL1403(pFL3) and ILCitM(pFL3).



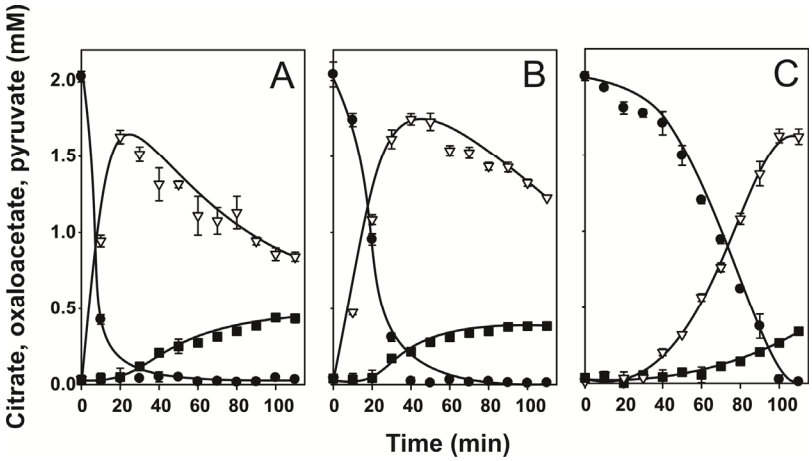
The internal pH (A) and the membrane potential (B) of cells of *L. lactis* IL1403(pFL3) (*wt*) and ILCitM(pFL3) (*mut*) were continuously monitored in time. At the times indicated by the arrows, 1 mM of L-lactate and 2 mM of citrate were added. The internal pH was evaluated from the pH dependent fluorescence of BCECF as described in the Materials and methods section. Changes in the membrane potential were qualitatively evaluated from the quenching of the potentiometric probe $DiSC_3$.

Following depletion of citrate, the concentration of oxaloacetate outside decreased again (Fig. 4A, B). At the same time, an increase in pyruvate and acetate was observed suggesting re-uptake of oxaloacetate by the cells and, subsequent, conversion in the cytoplasm. Oxaloacetic acid is known to be unstable, undergoing spontaneous decarboxylation to pyruvate. Control experiments in buffer demonstrated that under the conditions of the experiment the non-enzymatic reaction resulted in about 15 % of oxaloacetate converted to pyruvate in one hour (not shown), which was significantly slower than the decrease observed in the oxaloacetate concentration following the depletion of citrate. The results indicate that in spite of the CitM null allele, decarboxylation of oxaloacetate in the cytoplasm is still catalyzed producing the intermediate pyruvate that, in part, is excreted by the cells and, in part, is further metabolized to acetate.

Oxaloacetate is a substrate of CitP

Oxaloacetate is a divalent, negatively charge dicarboxylate that is not likely to cross the cytoplasmic membrane by passive diffusion. The similar kinetics of citrate degradation and oxaloacetate production by the mutant strain ILCitM(pFL3) under all conditions tested (Fig. 4) suggests that the excretion of oxaloacetate is coupled to the uptake of citrate by the citrate transporter CitP. Oxaloacetate uptake was studied in the parental strain IL1403(pFL3) and in the original strain IL1403 that does not contain the gene encoding the citrate transporter CitP. In the presence of the latter strain, oxaloacetate decreased at a rate that was comparable to the spontaneous decarboxylation observed in buffer (see above) (Fig. 5B). Apparently, though the cells contain the oxaloacetate decarboxylase enzyme and downstream enzymes for further metabolism, they are unable to take up oxaloacetate from the medium. In contrast, a significantly

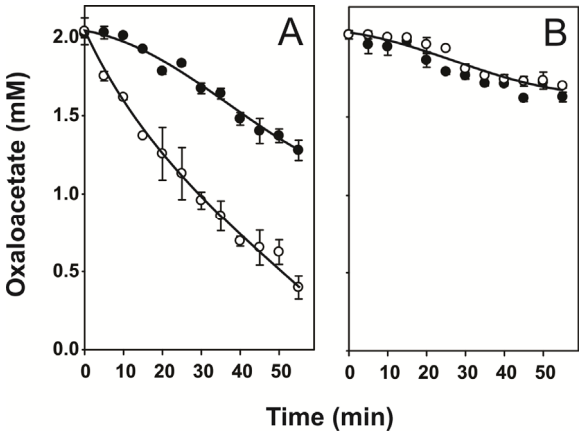
Figure 4. Products of the citrate metabolism in *L. lactis* ILCitM(pFL3).



A concentration of 2 mM citrate was added to the resting cells of *L. lactis* ILCitM(pFL3) in the presence of 0.1 mM glucose (A), 0.2 mM L-lactate (B), no further additions (C). Citrate consumption (●), and oxaloacetate (▽) and pyruvate (■) production were measured in time.

higher rate of oxaloacetate consumption was observed in strain IL1403(pFL3), containing the citrate transporter CitP. Similar to what was observed with citrate as the substrate (Fig. 2C), the decrease of oxaloacetate was biphasic with very little consumption during the first 10 minutes. Importantly, the rate of oxaloacetate consumption was enhanced in the presence of L-lactate, consistent with an oxaloacetate/L-lactate exchange catalyzed by CitP. In agreement, L-lactate did not have any effect on the decrease of the oxaloacetate concentration in the presence of strain IL1403 (Fig. 5B). The rate of oxaloacetate consumption by IL1403(pFL3) in the presence of L-lactate slowed down as the concentration of oxaloacetate decreased indicating a lower affinity of CitP for oxaloacetate than was observed for citrate (compare Fig. 2B and 5A).

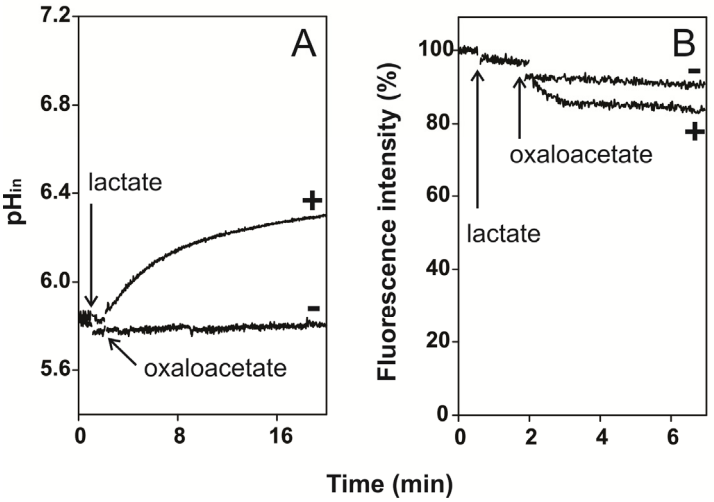
Figure 5. Oxaloacetate consumption in *L. lactis* IL1403 in the presence and absence of CitP.



A concentration of 2 mM oxaloacetate was added to the resting cells of *L. lactis* IL1403(pFL3) (A) and IL1403 (B) in the presence (○) and absence (●) of 1 mM L-lactate.

Oxaloacetate metabolism by *L. lactis* strain IL1403(pFL3) in the presence of 1 mM L-lactate resulted in the generation of a transmembrane pH gradient of 0.5 units (Fig. 6A). Similarly, a small, but significant membrane potential was generated (Fig. 6B). Cells of IL1403 lacking the CitP transporter showed no pmf generation upon the addition of oxaloacetate in the presence of L-lactate (Fig. 6). It follows that *L. lactis* IL1403(pFL3) metabolizes oxaloacetate by a similar mechanism as citrate.

Figure 6. Energetics of the oxaloacetate metabolism in *L. lactis* IL1403 and IL1403(pFL3).



The internal pH (A) and the membrane potential (B) of cells of *L. lactis* IL1403 lacking CitP (-) and IL1403 (pFL3) containing CitP (+) were continuously monitored in time. At the times indicated by the arrows, 1 mM of L-lactate and 2 mM of oxaloacetate were added. The cells were loaded with BCECF as described in the Materials and methods section. Changes in the membrane potential were qualitatively evaluated from the quenching of the potentiometric probe $DiSC_3$.

Discussion

Citrate metabolism in the mutant strain L. lactis ILCitM(pFL3)

The oxaloacetate deficient strain ILCitM(pFL3) was grown in batch culture at an initial pH of 7.0 in rich medium (M17) supplemented with 0.5% (w/v) glucose as carbon and energy source, and in the absence of citrate. The cells were harvested at mid-exponential growth phase where the pH had dropped to 6.8. Under these conditions, fermentation by the parent strain is homolactic (30) and citrate metabolic enzymes are present at levels that give high rates of citrate consumption in resting cells (23). Metabolism of citrate by resting cells of the mutant proceeds in two steps (Fig. 7A). The first step represents a short metabolic pathway in which citrate is converted to oxaloacetate involving the citrate transporter CitP and citrate lyase CL. Internalized citrate is converted by citrate lyase resulting in a rapid accumulation of oxaloacetate in the cytoplasm, which is then excreted in exchange with citrate. This step is ommitted in the parental strain IL1403(pFL3) as the activity of oxaloacetate decarboxylase prevents the accumulation of oxaloacetate in the cytoplasm. Citrate/oxaloacetate exchange performed by CitP represents a new example of substrate/intermediate exchange previously described by Pudlik and Lolkema (23). The rate of citrate/oxaloacetate exchange is as fast as citrate/L-lactate exchange and faster than exchange of citrate with other metabolic intermediates/products, which results in a more rapid consumption of citrate by the mutant than the parental strain when no L-lactate is present

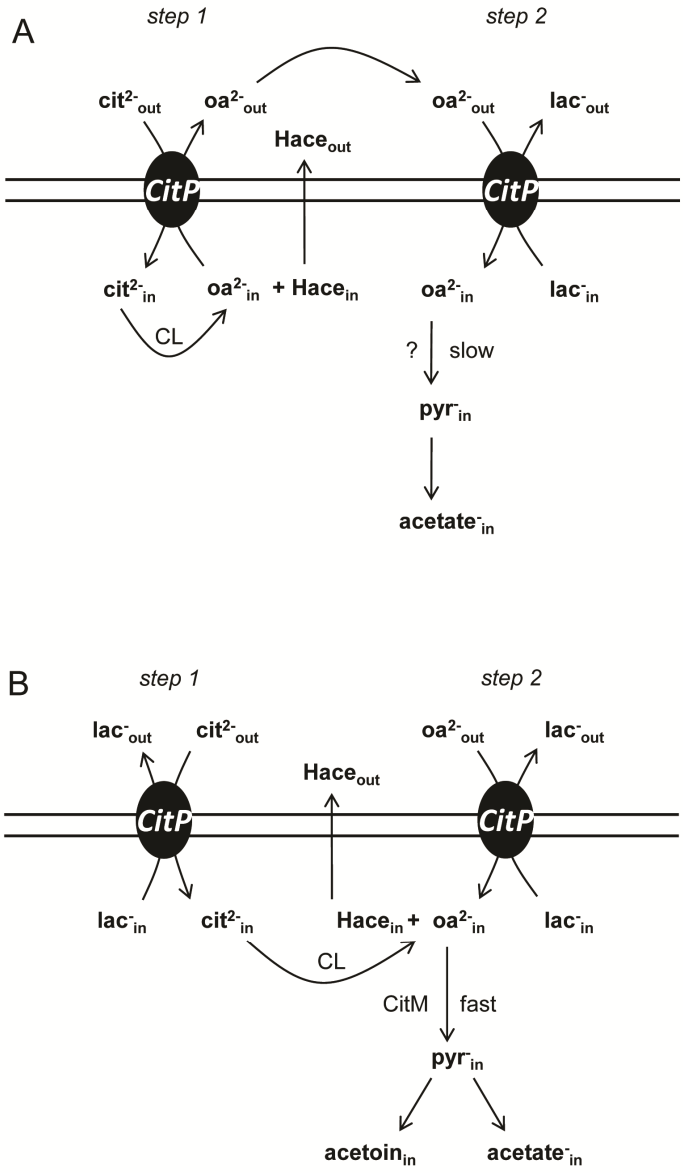
(Fig. 2C). Exchange of divalent citrate and divalent oxaloacetate is electroneutral and the proton produced in the CL reaction leaves the cell as acetic acid. Hence, the first step does not generate pmf. In the second step, oxaloacetate reenters the cell via CitP in exchange with an available intermediate/product to be slowly converted to the products pyruvate and acetate. No acetoin was formed under the conditions of the experiment, which is consistent with observations made in the parent strain showing that the conversion of pyruvate to acetate rather than acetoin was favored under condition when the flux through the pathway was low (Fig. 7B) (23). The low rate of the second step is determined by the slow decarboxylation of internalized oxaloacetate (see below). Uptake of divalent oxaloacetate in exchange with a monovalent weak acid, such as L-lactate, pyruvate or acetate, generates membrane potential even though this could not be demonstrated in the second phase of citrate consumption due to technical limitations. Together with proton consumption during oxaloacetate decarboxylation, the second step generates pmf.

The oxaloacetate deficient mutant mimics the physiological conditions of citrate fermenting *L. lactis* when oxaloacetate accumulates in the cells. Growth experiments showed reduced growth rates of the mutant and excretion of oxaloacetate in the medium suggesting toxic effects of high concentrations of the latter in the cytoplasm (1), probably arising from competitive inhibition of other enzymes. *L. lactis* responses to a slowing down of the oxaloacetate degradation rate by excreting oxaloacetate in exchange with citrate thereby making elegant use of the properties of CitP. Once the threat is over, i.e. all citrate is consumed, oxaloacetate is taken up again to be slowly further metabolized. Major consequences are the transient accumulation of oxaloacetate in the external medium, acetate is the major product, and the pathway is less efficient in generating pmf since no pmf is generated in the conversion of pyruvate to acetate (23).

Oxaloacetate metabolism in the parental strain L. lactis IL1403(pFL3)

The citrate transporter CitP catalyzes translocation of both divalent citrate and oxaloacetate across the cytoplasmic membrane in the same transport modes. Fast electrogenic oxaloacetate/L-lactate exchange was observed in the presence of L-lactate (Fig. 5A and 6A) and glucose (not shown) in the wild type strain. In the absence of L-lactate, oxaloacetate consumption was biphasic as observed for citrate consumption (Fig. 2C and 5A) indicating uptake in symport with a proton and subsequent exchange with a metabolic intermediate/product. Consequently, cells of *L. lactis* IL1403(pFL3) can metabolize oxaloacetate by a similar mechanism as citrate by simply bypassing the citrate lyase reaction (Fig. 7B). Transporters for oxaloacetate are rare in the prokaryotic domains of life. CitP is a member of the 2-hydroxycarboxylate transporter (2-HCT) family (28) (TC 2.A.24) (24). The 2-HCT family is believed to be distantly related to the divalent anion/Na^+ symporter family (DASS) (TC classification 2.A.47) (15, 24). The dicarboxylate transporters DccT of *Corynebacterium glutamicum* (31) and SdcL of *Bacillus licheniformis* (29) are found in the latter family and were shown to be competitively inhibited by oxaloacetate.

Figure 7. Transport and metabolism of citrate and oxaloacetate in *L. lactis*.



(A) Model of citrate uptake and metabolism in the mutant strain of *L. lactis* ILCitM(pFL3). (B) Model of citrate or oxaloacetate uptake and metabolism in the parental strain *L. lactis* IL1403(pFL3). cit, citrate; oa, oxaloacetate; pyr, pyruvate; ace, acetate; lac, L-lactate.

Cryptic oxaloacetate decarboxylase activity

In spite of the inactivation of oxaloacetate decarboxylase, oxaloacetate was still decarboxylated in the experiments with the mutant strain. Oxaloacetate has long been known to be unstable in aquous solution and to spontaneously decarboxylate to yield pyruvate (12). The rate of spontaneous decarboxylation in the buffers used in the present experiments was about 15% per

hour which was too slow to account for the oxaloacetate consumption rate by the mutant strain (Fig. 5). Also, the presence of the cells did not increase the rate as the same rate was observed in the presence of the wild type strain IL1403 that does not consume oxaloacetate (Fig. 5B). It follows that the oxaloacetate decarboxylation activity observed in *L. lactis* IL1403(pFL3) must be enzyme catalyzed.

Two classes of oxaloacetate decarboxylases are distinguished: (i) the membrane-bound sodium pumps termed OAD that have been well characterized in Gram-negative bacteria (9), and (ii), the less well characterized soluble enzymes from the large malic enzyme family that are mostly found in Gram-positive bacteria. Oxaloacetate decarboxylase of *L. lactis* encoded by the *mae* gene belongs to the second group and no OAD genes are encoded on the chromosome. The *mae* gene product of *L. lactis* is the only soluble oxaloacetate decarboxylase that has been characterized in detail (25). The deletion mutant was created by a frame shift deletion of 14 base pairs between position 584 and 598 in the gene (1). It is highly unlikely that the remaining gene product would fold into a stable enzyme that could account for the slow oxaloacetate decarboxylation activity observed in the mutant strain. Most likely, the activity is catalyzed by some other enzyme(s) in the cells as an unknown side reaction. Oxaloacetate decarboxylase activity of other enzymes has been reported. Examples would be pyruvate kinase (7) and some malic enzymes (MEs) (22) that are found to be encoded on the chromosome of *L. lactis* IL1403 (5).

Substrate specificity of CitP

Previous studies performed with rigth-side-out (RSO) membrane vesicles derived from *L. lactis* producing CitP of *Leuconostoc mesenteroides* showed that CitP has affinity for a wide range of physiological and non-physiological 2-hydroxycarboxylates, all sharing the motif 2-HC, $HO\text{-}CR_2\text{-}COO^-$. Substrates with the motif were all good substrates, however, also some 2-ketoacids, i.e. oxaloacetate and pyruvate, and 3-hydroxycarboxylates were transported by CitP with low efficiency (2, 4). The assay used in these studies was the capacity of different substrates to chase accumulated citrate from the mebranes and, therefore, assays for transport from ‘out’ to ‘in’. More recently, the ability of CitP to translocate efficiently exchange with the 2-ketoacid pyruvate and the acid acetate was demonstrated using a different experimental set up with whole cells that assays transport in the opposite direction, from ‘in’ to ‘out’ (‘shuttle’ mechanism) (23). Transport of acetate was not observed in the previous assay using RSO membranes suggesting that translocation by CitP in both directions is kinetically asymmetric. The assay employing whole cells requires that the substrates can diffuse across the cytoplasmic membrane passively, which limits the assay to monocarboxylates that are permeable in the protonated state. The present study demonstrates that the dicarboxylate 2-ketoacid oxaloacetate is a substrate of CitP as well. The high rate of exchange with citrate by the mutant strain (Fig. 7A, step 1) shows efficient translocation from ‘in’ to ‘out’. Consumption of oxaloacetate by the parental strain shows that the

affinity for transport in the ‘out’ to ‘in’ direction (step 2) is significantly lower than for the tricarboxylate, 2-hydroxy substrate citrate. It follows that CitP is a remarkable promiscuous transporter with affinity for mono, di, and tricarboxylates substituted or not at the C2 atom.

References

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Chapter 4

Substrate specificity of the citrate transporter CitP of *Lactococcus lactis*

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4

Abstract

The citrate transporter CitP of lactic acid bacteria catalyzes electrogenic precursor/product exchange of citrate *versus* L-lactate during citrate/glucose co-metabolism. In the absence of sugar, L-lactate is replaced by the metabolic intermediates/end products pyruvate, α-acetolactate or acetate. In this study, the binding and translocation properties of CitP were analyzed systematically for a wide variety of mono- and dicarboxylates of the form $X\text{-}CR_2\text{-}COO^-$, in which X represents OH (2-hydroxy acid), O (2-keto acid), or H (acid) and R groups differ in size, hydrophobicity, and composition. It follows that CitP is a very promiscuous carboxylate transporter. A carboxylate group is both essential and sufficient for recognition by the transporter. A C2 atom is not essential, formate is a substrate, and C2 may be part of a ring structure as in benzoate. The R group may be as bulky as an indole ring structure. For all monocarboxylates of the form $X\text{-}CHR\text{-}COO^-$, the hydroxy (X=OH) analogs were the preferred substrates. The preference for the keto (X=O) or acid (X=H) analogs was dependent on the bulkiness of the R group such that the acid was preferred for small R groups and the 2-ketoacids for more bulky R groups. The C4-6 dicarboxylates succinate, glutarate, and adipate were also substrates of CitP. The broad substrate specificity is discussed in the context of a model of the binding site of CitP. Many of the substrates of CitP are intermediates or products of amino acid metabolism suggesting that CitP may have a broader physiological function than in citrate fermentation alone.

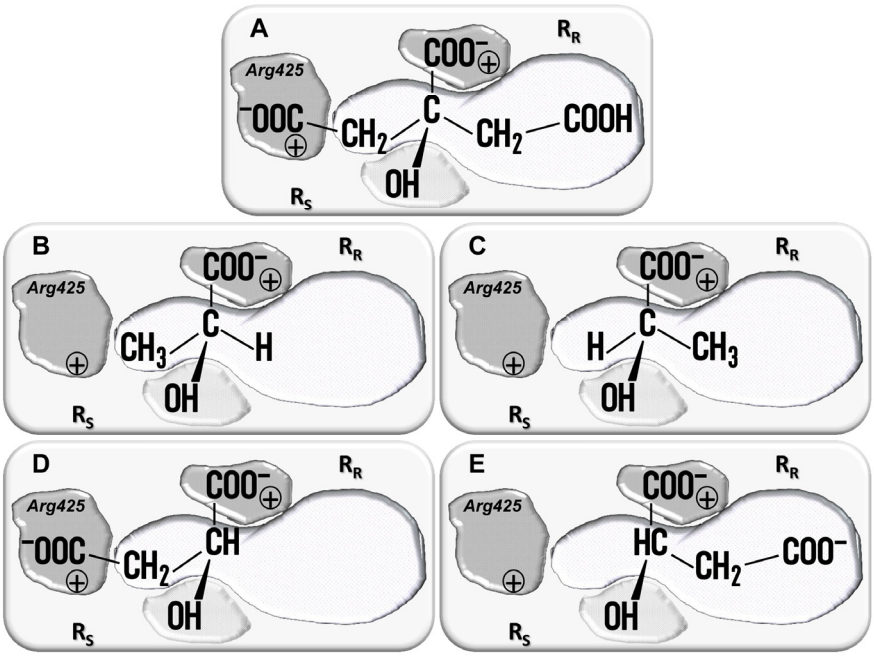
Introduction

The citrate transporter CitP functions in citrate fermentation by Lactic Acid Bacteria (LAB) like *Lactococcus lactis* and *Leuconostoc mesenteroides* (16, 18). Internalized citrate is split into acetate and oxaloacetate, after which the latter is decarboxylated yielding pyruvate. During co-metabolism with glucose, pyruvate is reduced to L-lactate (9, 12, 14). CitP catalyzes uptake of divalent citrate ($Hcit^{2-}$) in exchange with monovalent L-lactate (lac^-) (precursor/product exchange), which results in generation of membrane potential (Δψ) (13, 14, 15). Together with proton consumption in decarboxylation reactions in the citrate metabolic pathway that results in a transmembrane pH gradient (ΔpH), the pathway generates proton motive force (pmf) (10, 11, 16). Recognition by CitP of two structurally related but different substrates, i.e. the tricarboxylate citrate and monocarboxylate L-lactate suggests an inherent broad substrate specificity of the transporter.

In vitro citrate transport studies using right-side-out (RSO) membrane vesicles derived from *L. lactis* demonstrated that CitP has affinity for 2-hydroxycarboxylates of the form $HO\text{-}CR_2\text{-}COO^-$, in which the R group ranged from a hydrogen atom in glycolate to a phenyl group in mandalate and to acetyl groups in malate and citrate (2). The transporter was shown to discriminate between high affinity substrates that contain a second carboxylate group in one of the R substituents like citrate and malate, and low affinity substrates, monocarboxylates like lactate, suggesting an important role of the second carboxylate group in the interaction with the protein. Based on the experiments, a model of the binding site of CitP was proposed (Fig. 1), in which the carboxylate and hydroxyl group of the 2-hydroxycarboxylate (2-HC) motif present in all substrates interact with specific sites on the protein (2, 3). This would fix the orientation of the substrate in the binding pocket and define two separate sites in the binding pocket (R_S and R_R) (Fig. 1) for optional interactions with the R groups of the substrates, including the interaction with a second carboxylate in the R_S site that results in high affinity binding. In agreement, the (S)-enantiomers of chiral dicarboxylate substrates like malate were bound with high affinity and the (R)-enantiomers with low affinity whereas both enantiomers of monocarboxylates like lactate were low affinity substrates (3). Site directed mutagenesis of CitP of *L. mesenteroides* identified the conserved Arg425 residue as the site specifically interacting with the second carboxylate present in (S)-divalent substrates (4). Additionally, it was observed that increasing binding affinity of monocarboxylates with increasing hydrophobicity of the R groups suggested a hydrophobic nature of the R_R and R_S sites. Evidence was put forward that at least part of the sites are located in the C-terminal 46 residues (3).

While the carboxylate group of the 2-HC motif was essential for the interaction of CitP with a substrate, the transport studies in RSO membranes showed that the hydroxyl group could be replaced to some extent by keto groups, i.e. in oxaloacetate and pyruvate (2). Experiments in whole cells of *L. lactis* expressing the citrate fermentative pathway confirmed the affinity of CitP for these two metabolic intermediates of the pathway and also demonstrated the physiological relevance. In the absence of L-lactate, CitP catalyzes uptake of citrate in exchange with

Figure 1. Schematic model of the substrate binding pocket of CitP.



Substrates depicted in the pocket are citrate (A), (S)-lactate (B), (R)-lactate (C), (S)-malate (D), and (R)-malate (E). The interactions between the carboxylate groups and the hydroxyl group of the substrate and the protein were indicated as grey surfaces, the hydrophobic interaction site (R_R) by a white surface. The R_S and R_R sites bind the side chains of the (S)- and (R)-enatiomers of monosubstituted 2-hydroxycarboxylates (HO-CHR-COO^-), respectively. Residue Arg425 responsible for binding of a carboxylate in the R side chain, when present, was indicated in the R_R site.

oxaloacetate and/or pyruvate when these accumulate in the cytoplasm. In addition to these two intermediates, citrate could also be taken up in exchange with acetate, the end product of the pathway showing that CitP can bind and translocate substrates in which the hydroxyl group at the C2 atom is replaced by a keto group or a hydrogen atom (18, 19).

Here, a systematic study of the substrate specificity of the citrate transporter CitP of LAB is presented. A wide range of analogous substrates differently substituted at the C2 atom and with various R group, both monocarboxylates and dicarboxylates, were included. It follows that CitP is a remarkably promiscuous transport protein. Many of the substrates of CitP are secondary metabolites derived from amino acid metabolism and important as flavor compounds or precursors thereof in food fermentations.

Materials and methods

Chemicals

2-hydroxy-4-methylthiobutyrate, 2-keto-4-methylthiobutyrate, 4-methylthiobutyrate, acetate, adipate, benzoate, formate, fumarate, glutarate, glycolate, glyoxylate, indole-3-lactate, indole-3-

propionate, indole-3-pyruvate, isocaproate, isovalerate, L-lactate, L-malate, maleate, malonate, oxalate, oxaloacetate, phenylacetate, phenylglycolate, phenylglyoxylate, phenyllactate, phenylpropionate, phenylpyruvate, propionate, pyruvate, succinate, α-hydroxyisocaproate, α-hydroxyisovalerate, α-ketoisocaproate, α-ketoisovalerate were obtained from Sigma Aldrich. L-lactate dehydrogenase (L-LDH), L-malate dehydrogenase (L-MDH), and citrate lyase (CL) were obtained from Roche Applied Science. 2',7'-bis-(2-carboxyethyl)-5(and-6)-carboxyfluorescein (BCECF, acid form) and 3,3'-dipropylthiocarbocyanine iodide ($DiSC_3$) probes were obtained from Invitrogen Molecular Probes.

Bacterial strain and growth condition

Strain *Lactococcus lactis* IL1403(pFL3) was used in this study. Plasmid pFL3 harbours the lactococcal CRL264 *citP* gene under control of the *Streptococcus pneumoniae polA* promoter (12). Neither expression nor plasmid copy number are under the control of citrate or pH in this strain (8). Precultures were grown overnight at 30 ^{o}C in M17 broth medium supplemented with 0.5 % (w/v) glucose (M17G) and 5 μg.ml^{-1} of tetracycline. Cells were grown in M17G medium with an initial pH adjusted to 7.0. Growth was performed in 100 ml serum bottles without agitation and at 30 ^{o}C. Growth was followed by measuring the optical density at a wavelength of 660 nm. Cells were harvested at mid-exponential growth phase when the optical density was 0.6 by spinning for 10 min at 3000 rpm. Cells were washed two times with 50 mM potassium phosphate pH 5.8 buffer, and finally, resuspended in the same buffer at 4 ^{o}C.

Citrate consumption by resting cells of L. lactis IL1403(pFL3)

Resting cells at an OD_{660} of 1.5 in 50 mM potassium phosphate pH 5.8 buffer were incubated at 30 ^{o}C without agitation for 10 min. The assay was performed in a total volume of 1.5 ml. At t=0, citrate was added at a concentration of 2 mM. When indicated, additional substrates were added from 10-fold stock solutions set at pH 5.8 together with citrate. The pH of the suspension was monitored after the experiment and never changed by more than 0.1 unit. Samples of 100 μl were taken every 5 or 10 min and immediately centrifuged for 0.5 min at maximum speed in a table top centrifuge. The supernatant was stored on ice until further analysis by enzymatic assays. Initial rates of citrate consumption were calculated from the decrease in citrate concentration within the first 10 min, assuming zero-order kinetics.

Enzymatic assays

Citrate, oxaloacetate, and pyruvate were measured as described before (18), using the commercially available enzymes citrate lyase (CL), L-malate dehydrogenase (L-MDH), and L-lactate dehydrogenase (L-LDH). Briefly, an aliquot of 30 μl of the sample was added to 50 mM glycine-glycine pH 7.8 buffer containing NADH and L-MDH. Oxaloacetate in the sample is converted to L-malate at the expense of NADH. Subsequently, pyruvate in the same sample was

measured by addition of L-LDH which results in the conversion of pyruvate to L-lactate at the expense of NADH. Subsequent addition of CL converts citrate in the sample to oxaloacetate (and pyruvate) resulting in an additional decrease in the NADH concentration equivalent to the citrate concentration present in the samples. The assay was performed in 96-well microtiter plates. The decrease in NADH concentration was measured spectroscopically at 340 nm. Standard deviations were calculated from three independent experiments.

Measurement of internal pH (ΔpH) and membrane potential (ΔΨ)

The components of the proton motive force were measured as described before (18). To measure ΔpH, resting cells resuspended to high density (typically containing 50 mg/ml of protein) in 50 mM potassium phosphate pH 5.8 buffer were loaded with BCECF. Fluorescence measurements were performed in 1 cm cuvettes containing 50 mM potassium phosphate pH 5.8 buffer equilibrated at 30 ^{o}C and cells loaded with BCECF. The cuvette was stirred with a magnetic stirring bar. Fluorescence was measured using excitation and emission wavelengths of 502 and 525 nm, respectively, with slit widths of 4 and 16 nm, respectively. The fluorescence signal was sampled every second. Opening of the measurement compartment caused loss of data during the first 5-6 seconds after an addition to the cuvette was made. The cytoplasmic pH was calculated as described by (17).

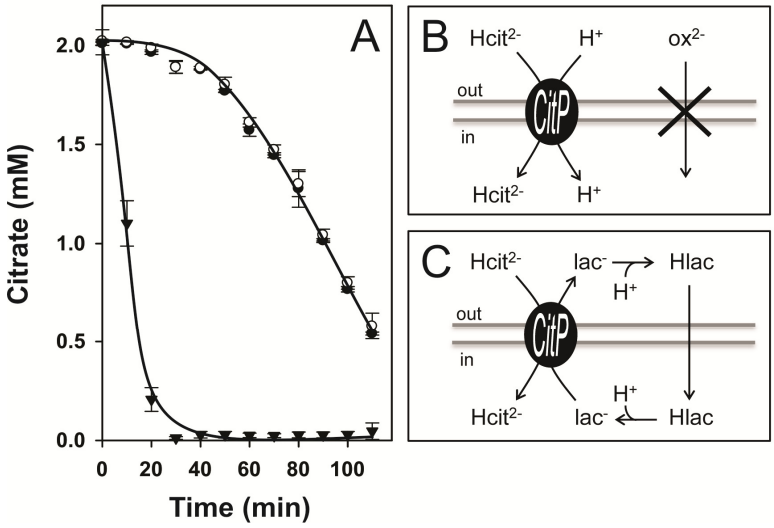
Membrane potential was measured qualitatively with the fluorescent probe $DiSC_3$ (22). Decrease in fluorescence intensity correlates with an increase in electrical potential across the membrane. $DiSC_3$ was added from a stock solution to a final concentration of 2 μM to the quartz cuvettes containing with 50 mM potassium phosphate pH 5.8 buffer and cells. The system was left to equilibrate for 5 min at 30 ^{o}C. Fluorescence measurements were performed using excitation and emission wavelengths of 500 and 705 nm, respectively, and slit widths of 8 nm, respectively.

Results

Substrate specificity assay for CitP

L. lactis strain IL1403(pFL3) harbors plasmid pFL3 that contains the citrate transporter gene *citP* under control of the constitutive *Streptococcus pneumoniae polA* promoter (12). The genes encoding the metabolic enzymes of the citrate fermentation pathway are present on the chromosome of the IL1403 host strain (5). Cells of *L. lactis* IL1403(pFL3) grown in M17 broth medium supplemented with 0.5 % of glucose till the mid-exponential growth phase (OD_{660}=0.6) were resuspended in potassium phosphate pH 5.8 buffer at OD_{660}=1.5. Following addition of 2 mM of citrate, the consumption of citrate showed to be biphasic (Fig. 2A) (18). The first phase represents slow uptake of citrate coupled to a H^+ (H^+/$Hcit^{2-}$ symport) (Fig. 2B). The second phase represents fast uptake of citrate in exchange with intermediates/end products of citrate metabolism in the cytoplasm, i.e. pyruvate, α-acetolactate, and acetate, which accumulate in the

Figure 2. Substrate specificity assay for CitP.



A. Citrate consumption by resting cells of *L. lactis* IL1403(pFL3) in the presence of 0.2 mM L-lactate (▼), 2 mM oxaloacetate (○), and no further additions (●). B. Schematic of transport of citrate by CitP operating in the H^+-symport mode in the presence of none permeative dicarboxylates. Oxaloacetate (ox^{2-}) cannot enter the membrane by passive diffusion, but competes with citrate ($Hcit^{2-}$) at the outside of the cell. C. Schematic of the shuttle mechanism in the presence of permeative monocarboxylates. L-Lactate added at the outside of the cells allows CitP to operate in the fast $Hcit^{2-}/lac^-$ exchange mode by reentering the cell in the permeative protonated state (Hlac).

cell during the first phase. Both modes of transport are catalyzed by the citrate transporter CitP (18). In the presence of 0.2 mM L-lactate, a physiological substrate of CitP, the first slow phase is skipped and citrate is taken up in fast exchange with internal L-lactate from the beginning (Fig. 2A). The mechanism of uptake was described before as the shuttle mechanism (14, 18) (Fig. 2C). The acceleration of citrate consumption by the addition of a compound can be used to identify substrates of CitP when two conditions are met: (i) recognition of the substrate by CitP in the cytoplasm and (ii) high permeability of the substrate through the membrane. The latter requirement is demonstrated when L-lactate was replaced by oxaloacetate, another substrate of CitP (19). No acceleration of consumption was observed because oxaloacetate cannot enter the cell (Fig. 2A, B). In addition, oxaloacetic acid does not compete with citrate at the outside because CitP has a much higher affinity for the latter (19). Monocarboxylates are weak acids that are permeative in the protonated state which makes them good candidates for the substrate specificity assay. Dicarboxylates may not be membrane permeable at physiological pH and substrates of CitP are only detected when an appropriate transporter is present in the membrane that supports a rate of uptake that can maintain the exchange reaction catalyzed by CitP. Apparently, the membrane of *L. lactis* does not contain such a transporter for oxaloacetate.

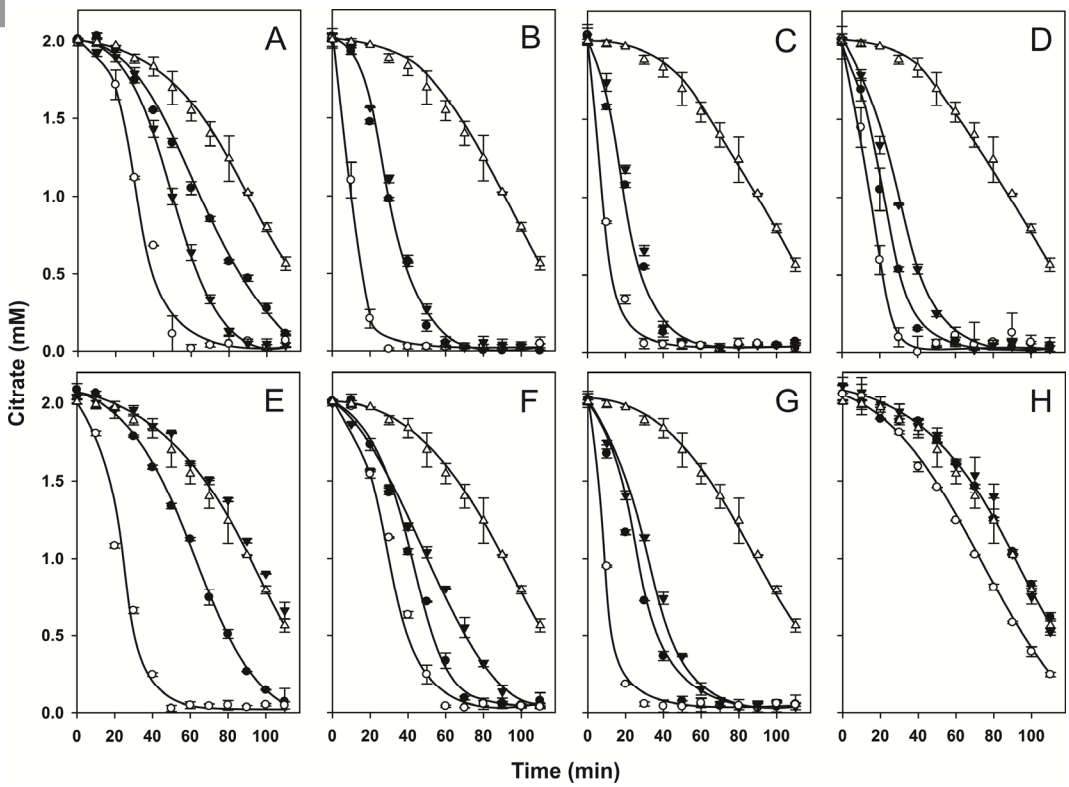
C2 substituted monocarboxylates

A set of 24 C2 substituted monocarboxylates of the form $X\text{-}CHR\text{-}COO^-$ were selected containing 8 different R groups and for each of these X either OH (hydroxy), O (keto), or H (acid). The 8 R groups correspond to the side chains of the 7 natural amino acids ($X=NH_3^+$), i.e. glycine, alanine,

valine, leucine, methionine, phenylalanine, and tryptophan, and the unnatural amino acid phenylglycine. The side chains differ in composition, size, and hydrophobicity (see Table S1). The selection results in 8 groups of C2 substituted analogs, f.i. glycolate, glyoxylate, and acetate derived from glycine, or phenyllactate, phenylpyruvate, and phenylpropionate derived from phenylalanine. All compounds were added at a concentration of 2 mM in the substrate specificity assay for CitP (Fig. 3).

All 8 hydroxy analogs increased the rate of citrate consumption significantly and in all cases more than observed with the keto and acid analogs (Fig. 3). Surprisingly, exchange of citrate and the OH-analogs of valine (α-hydroxyisovalerate), leucine (α-hydroxyisocaproate), and phenylalanine (phenyllactate) was as fast as observed for the physiological substrate L-lactate, the OH-analog of alanine (Fig. 3C, D, G, and B, respectively). These substrates supported the depletion of 2 mM citrate in 20-30 min. The OH-analogs of glycine (glycolate), methionine (2-hydroxy-4-

Figure 3. Transport of hydroxy, keto, and acid analogs of amino acids by CitP.



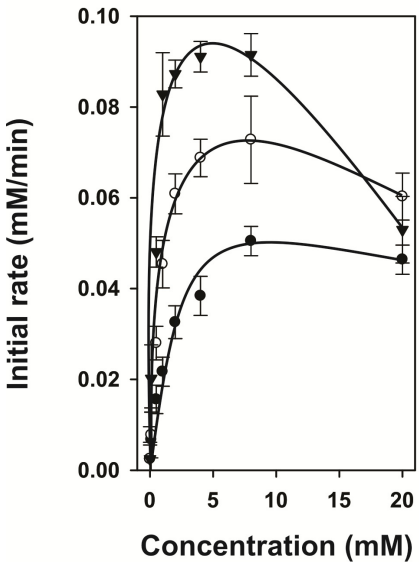
Citrate consumption by resting cells of *L. lactis* IL1403(pFL3) in the absence (Δ) and the presence 2 mM of the OH- (○), O- (●), and H- (▼) analogs of glycine (A): glycolate (○), glyoxylate (●), and acetate (▼); alanine (B): L-lactate (○), pyruvate (●), and propionate (▼); valine (C): α-hydroisoxyvalerate (○), α-ketoisovalerate (●), and isovalerate (▼); leucine (D): α-hydroxyisocaproate (○), α-ketoisocaproate (●), and isocaproate (▼); methionine (E): 2-hydroxy-4-methylthiobutyrate (○), 2-keto-4-methylthiobutyrate (●), and 4-methylthiobutyrate (▼); phenylglycine (F): phenylglycolate (○), phenylglyoxylate (●), and phenylacetate (▼); phenylalanine (G): phenyllactate (○), phenylpyruvate (●), and phenylpropionate (▼); and tryptophan (H): indole-3-lactate (○), indole-3-pyruvate (●), and indole-3-propionate (▼) (see Table S1).

methylthiobutyrate), and phenylglycine (phenylglycolate) resulted in a two times slower consumption, depletion within 50-60 min (Fig. 3A, E, and F, respectively), and the kinetics was biphasic. The OH-analog of tryptophan clearly showed the smallest effect, but the stimulation was significant indicating that also indole-3-lactate is a substrate of CitP (Fig. 3H). Seven out of eight of the keto analogs and six out of eight of the acid analogs were positively identified as substrates of CitP in the assay. Both the O-analog and H-analog of tryptophan (indole-3-pyruvate and indole-3-propionate, respectively) and the H-analog of methionine (4-methylthiobutyrate) did not affect the citrate consumption pattern (Fig. 3H and E, respectively). The H-analog resulted in a faster consumption of citrate than the O-analog for the glycine analogs, acetate and glyoxylate, respectively (Fig. 3A), while the rates were the same for the alanine (pyruvate and propionate) and valine (α-ketoisovalarate and isovalerate) analogs (Fig. 3B and C). However, in most cases, exchange with the O-analogs was faster than with the H-analogs (Fig. 3D, E, F, G, and H).

Relative activities with OH-, O-, and H-analogs

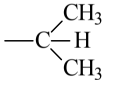
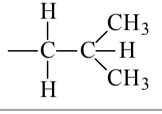
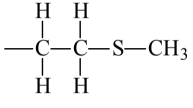
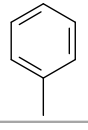
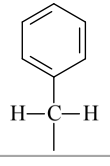
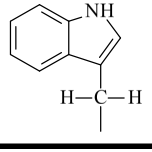
The difference between the hydroxy (OH), keto (O), and acid (H) analogs was further detailed by measuring the consumption rates of citrate in the presence of a range of concentrations between 0 and 20 mM of the three leucine analogs, i.e. α-hydroxyisocaproic acid, α-ketoisocaproic acid, and isocaproic acid. At a concentration of 2 mM the differences were relatively small (Fig. 3D). The initial rates of citrate consumption at increasing external concentration showed the same pattern for the three analogs (Fig. 4). The rates increased to reach a maximum value followed by a decrease of the rates at higher concentrations. The initial rise is the consequence of the increased rate of uptake of citrate in exchange with the analog, the maximum represents the maximal rate of the shuttle consisting of the diffusion of the analog through the membrane and the citrate/analog exchange steps, and the inhibition relates to competition of citrate and the analog at the outer face of the membrane. The initial rise was faster in the order H<O<OH suggesting the highest affinity of the cytoplasmic binding site of CitP for the OH-analog followed by the O-analog and the H-analog. Similarly, the maximal consumption rates increased from 0.051 to 0.073 to 0.092 mM.min^{-1} for the H-, O- and OH-analog, respectively, suggesting the same order H<O<OH in the maximal exchange

Figure 4. Effect of C2 substituents on transport via CitP in *L. lactis* IL1403 (pFL3).



Rates of citrate consumption in the presence of α-hydroxyisocaproic acid (▼), α-ketoisocaproic acid (○), and isocaproic acid (●) at the concentrations ranging from 0 to 20 mM.

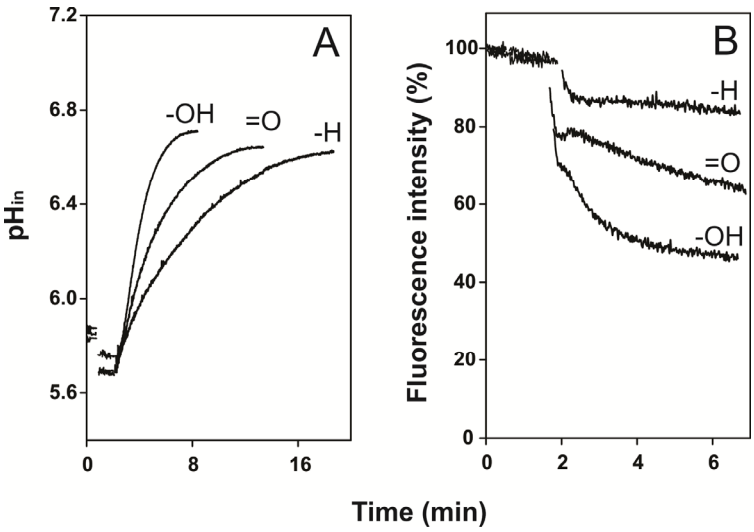
Supplementary Table S1. C2-substituted monocarboxylate substrates X-CHR-COO^-.

Substrate	X	R	$X=NH_3^+$ aa analog	Hydrophobicity of R (7)	Size of R	pK
glycolate	OH		glycine	0.48	-1.90	3.82
glyoxylate	O					3.32
acetate	H					4.76
lactate	OH		alanine	0.62	-1.46	3.86
pyruvate	O					2.50
propionate	H					4.87
α-hydroisoxyvalerate	OH		valine	1.08	-0.59	
α-ketoisovalerate	O					
isovalerate	H					4.77
α-hydroxyisocaproate	OH		leucine	1.06	-1.16	
α-ketoisocaproate	O					
isocaproate	H					4.84
2-hydroxy-4-methylthiobutyrate	OH		methionine	0.64	-1.16	
2-keto-4-methylthiobutyrate	O					
4-methylthiobutyrate	H					
phenylglycolate	OH		phenylglycine			3.41
phenylglyoxylate	O					2.15
phenylacetate	H					4.31
phenyllactate	OH		phenylalanine	1.19	1.15	
phenylpyruvate	O					
phenylpropionate	H					4.66
indole-3-lactate	OH		tryptophan	0.81	2.45	
indole-3-pyruvate	O					
indole-3-propionate	H					4.77

rates catalyzed by CitP. Also, the inhibition at higher concentrations increased in the order H<O<OH, suggesting increasing affinity for the analogs of the external binding site of CitP in the same order. It follows that for the leucine analogs α-hydroxyisocaproate is the best substrate of CitP both with respect to turnover in the citrate/analog exchange mode and the affinity of the transporter at the two sides of the membrane. Next best would be the keto analog, α-ketoisocaproate, followed by the acid analog, isocaproate.

Flux through the citrate metabolic pathway generates proton motive force both as pH gradient (ΔpH) and membrane potential (ΔΨ) (14, 18). The transmembrane pH gradient evaluated by measurement of the internal pH inferred from the fluorescent dye BCECF (17) and the membrane potential measured qualitatively by the potentiometric probe $DiSC_3$ (22) were measured in the presence of the three leucine analogs in potassium phosphate pH 5.8 buffer. Previously it was shown that the pH and membrane potential probes had an inhibitory effect on the flux through the citrate pathway in strain *L. lactis* IL1403(pFL3), which in part could be compensated for by increasing the concentration of the exchanged substrate (18). Addition of 2 mM citrate in the presence of 8 mM α-hydroxyisocaproic acid, the best leucine analog, resulted in the same ΔpH of 0.9-0.95 units as observed in the presence of 2 mM of the physiological substrate L-lactate (Fig. 5A) (18). Similarly, using the same conditions, a ΔΨ of comparable magnitude was generated (Fig. 5B) (18). In the presence of both 2-hydroxy-monocarboxylates, the pH gradient and membrane potential developed during the first 5 minutes of citrate consumption (Fig. 5) that proceeded at more or less the same rate (Fig. 4 and 6C). The O- and H-analog of leucine, i.e. α-ketoisocaproic acid and isocaproic acid, resulted in similar pH gradients of 0.85-0.9 units but the steady state was reached slower correlating with the slower consumption rates in the presence of the two analogs (Fig. 4 and 5A). In contrast, the extent of the membrane potential seemed to

Figure 5. Effect of C2 substituents on generation of ΔpH (A) and Δψ (B) by citrate metabolism in *L. lactis* IL1403(pFL3).



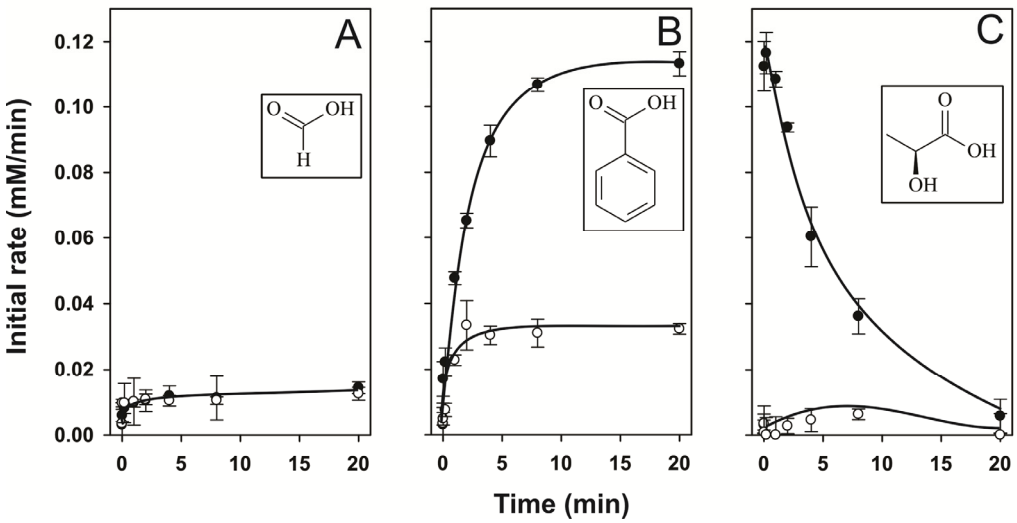
The internal pH of the cells loaded with BCECF (see Materials and methods) was continuously monitored in time (A) and changes in the membrane potential were qualitatively evaluated from the quenching of the potentiometric probe $DiSC_3$ (see Materials and methods) (B). At the times indicated by the arrows, 8 mM of α-hydroxyisocaproic acid (-OH), α-ketoisocaproic acid (=O), or isocaproic acid (-H) (A) was added. Citrate was added at a concentration of 2 mM.

decrease in the order OH>O>H (Fig. 5B). It follows that the energetics of citrate consumption was the same in the presence of the three leucine analogs and the physiological substrate L-lactate. The pmf increased in the order H<O<OH. Moreover, it seems that the extent of the membrane potential is more sensitive to the flux through the pathway than the pH gradient.

Activity with the monocarboxylates formic acid and benzoic acid

Formate does not have a C2 atom and the C2 atom of benzoate is part of the aromatic phenyl ring. The cell membrane is very permeable to the protonated forms of both that have pK's values of 3.8 and 4.2, respectively. Titration of the citrate consumption rate with increasing concentrations of formate in the range of 0-20 mM revealed only a small maximal acceleration of the initial rate to 0.014 mM.min^{-1} that was obtained at relatively low concentrations (<1 mM) (Fig. 6A). By comparison, at 0.2 mM of the physiological substrate L-lactate, the rate was ten times higher (0.12 mM.min^{-1}) (Fig. 6C). It demonstrates that while CitP has affinity in the sub milimolar for cytoplasmic formate, the translocation rate of citrate/formate exchange is relatively slow. Benzoic acid proved to be a much better substrate of CitP (Fig. 6B). With increasing concentrations of benzoic acid added, the rate of citrate consumption increased to up to 0.11 mM.min^{-1}, very close to the maximal rate of 0.12 mM.min^{-1} observed with L-lactate. However, the maximum rate of the uptake in the presence of benzoate was observed at a 1000-fold higher concentration than was the case for L-lactate, i.e. at 20 mM and 0.02 mM, respectively (Fig. 6B and C) (see also 18). It follows that while the rate of citrate/benzoate exchange is fast, the affinities of CitP at both sides of the membrane for the benzoate anion are low. The different affinities affect the product profile of

Figure 6. Formate and benzoate are substrates of CitP.



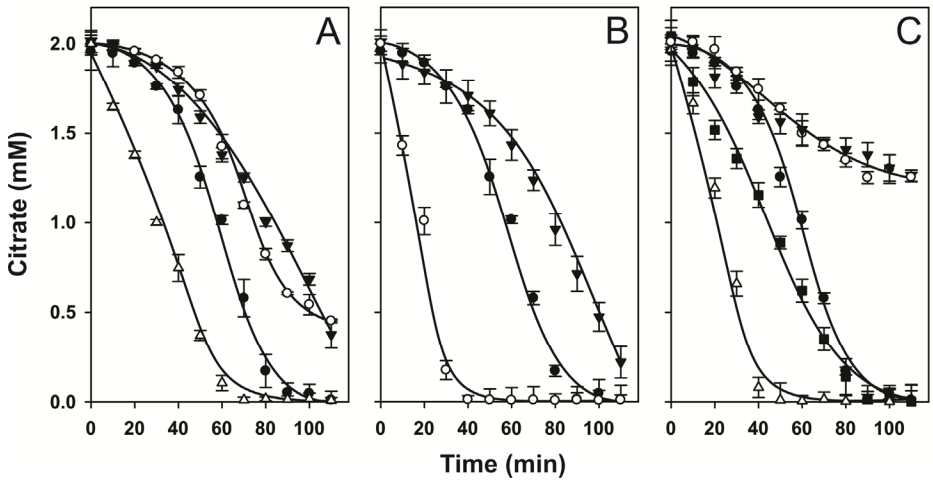
Rates of citrate consumption (●) and pyruvate production (○) by cells of *L. lactis* IL1403(pFL3) in the presence of formic acid (A), benzoic acid (B), and lactic acid (C) at concentrations ranging from 0-20 mM.

the citrate pathway in the cells. The high affinity of CitP for L-lactate ensures that the transporter remains in the citrate/L-lactate exchange mode during complete consumption. The intermediate pyruvate is not excreted (Fig. 6C). In contrast, CitP has higher affinity for pyruvate than for benzoate, and the transporter switches rapidly from citrate/benzoate exchange in the initial stages to citrate/pyruvate exchange once enough cytoplasmic pyruvate is produced. Pyruvate shows up as a major product under these conditions (Fig. 6B).

Transport of dicarboxylates by CitP

The C4-dicarboxylates L-malate, oxaloacetate, and succinate represent a series of analogs of the hydroxy (OH), keto (O), and acid (H) form (see Table S2), respectively, as introduced above for the monocarboxylates. Significant effects on citrate consumption by the cells were observed only at higher concentrations of these compounds. The presence of 2 mM citrate and 32 mM of L-malate or oxaloacetate resulted in a slight inhibition of the consumption rate which is due to inhibition of citrate uptake by the analogs at the outside of the membrane, i.e. competition between citrate and L-malate and between citrate and oxaloacetate (Fig. 7A, see Fig. 2B). Surprisingly, the H-analog succinate enhanced the consumption of citrate significantly (Fig. 7A) identifying succinate as a substrate of CitP. The high concentration necessary to see the acceleration may reflect a low affinity of CitP for internal succinate, but is more likely related to low permeability of succinate resulting in a low cytoplasmic concentration (see Fig. 2C). Transport of succinate into the cell probably relies on a transporter protein in the membrane. The double bond between C2 and C3 in the geometric isomers maleic acid (*cis*) and fumaric acid (*trans*) fixes the

Figure 7. Transport of dicarboxylates by CitP.



A. Effect of C2 substituent in dicarboxylates on transport via CitP. Citrate consumption by resting cells of *L. lactis* IL1403(pFL3) in the absence (●) and the presence of (A) 32 mM of the C4 dicarboxylates L-malate (○), oxaloacetate (▼), and succinate (Δ), (B) 32 mM of fumaric acid (▼) or maleic acid (○), and (C) 32 mM of oxalic acid (○), malonic acid (▼), glutaric acid (Δ), and adipic acid (■).

relative positions of the two carboxylate groups of succinate in space. In the presence of 32 mM of the *cis* isomer maleate the consumption rate was faster than in the presence of the same concentration of succinate and approached the rate observed with 2 mM of the physiological substrate L-lactate. In marked contrast, 32 mM of the *trans* isomer fumarate did inhibit citrate consumption (Fig. 7B). The length of the carbon chain in the dicarboxylates appeared to play an important role in the ability of the substrate to enhance citrate metabolism (Table S2). Oxalate (C2) and malonate (C3) inhibited the rate of citrate consumption to some extent, while succinate (C4), glutarate (C5), and especially adipate (C6) increase the rate (Fig. 7C). It follows that the dicarboxylates used here affect the rate of citrate consumption, either by inhibition (malate, oxaloacetate, fumarate, oxalate, and malonate) or acceleration (succinate, maleate, glutarate, and adipate) and, therefore, are recognized by CitP.

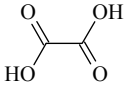
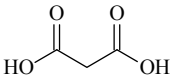
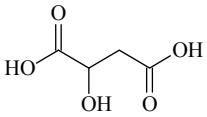
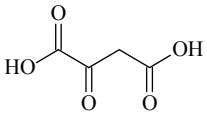
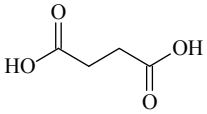
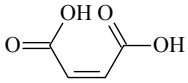
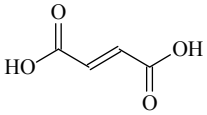
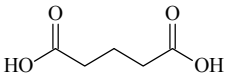
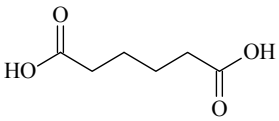
4

Discussion

Substrate specificity of CitP

The citrate transporter CitP is a member of the 2-hydroxycarboxylate transporter (2-HCT) family (Transporter Classification (TC) 2.A.24) (21), in which also the malate-lactate exchanger MleP of lactic acid bacteria, the Na^+-citrate symporter CitS of *Klebsiella pneumonia*, the H^+-malate/citrate symporter CimH of *Bacillus subtilis*, and the H^+-malate symporter MalP of *Streptococcus bovis* are found (24). Substrates of the transporters share the 2-hydroxycarboxylate (2-HC) motif, i.e. HO-CR_2-COO-, hence the name of the family (2). The functionally characterized symporters in the family have narrow substrate specificity while the exchangers have a much broader specificity which is inherent to their physiological function of exchanging precursor and product of a metabolic pathway. Previous and the present studies have demonstrated that substrates of CitP are not restricted to 2-hydroxycarboxylates even though these appear to be the best substrates. CitP is best characterized as a very promiscuous carboxylate transporter. A C2 atom is not essential (formate) or may be part of a delocalized ring structure (benzoate). Most substrates of CitP take the form X-CR_2-COO- in which X is either OH, O, or H but not NH_3^+ (amino acids). CitP is very tolerant towards the R groups. At the lower size limit H atoms are accepted making glycolate, glyoxylate, and acetate substrates. At the higher size limit, the binding pocket seems to discriminate between hydrophilic and hydrophobic R groups allowing more bulky groups in case of the latter. Then, isocitrate is not a substrate, while the 5 plus 6 ringed indole group is (2) (Fig. 3H). A number of the substrates other than 2-hydroxycarboxylates have been shown to be of physiological importance in the citrate fermentation pathway (18, 19). In addition, the broad specificity may be related to a function of the transporter in degradative amino acid pathways (see below).

Supplementary Table S2. Dicarboxylate substrates of CitP.

Common name	Number of C atoms	OH substituent	Structural formula	pK
Oxalate	2	-		(1) 1.23 (2) 4.19
Malonate	3	H		(1) 2.83 (2) 5.69
Malate	4	OH		(1) 3.40 (2) 5.11
Oxaloacetate	4	O		(1) 2.22 (2) 3.89
Succinate	4	H		(1) 4.16 (2) 5.61
Maleate	4 (cis)	H		(1) 1.83 (2) 6.07
Fumarate	4 (trans)	H		(1) 3.03 (2) 4.44
Glutarate	5	H		(1) 4.31 (2) 5.41
Adipate	6	H		(1) 4.43 (2) 5.41

CitP specificity assay

The transport studies using RSO membranes reported before determined the (kinetic) affinity of CitP for substrates at the external face of the membrane in the exchange reaction (4). The citrate consumption studies using resting cells reported here identify substrates recognized by CitP at the cytoplasmic face of the membrane. The substrate, f.i. L-lactate, enters the cell by a CitP-independent mechanism, after which the substrate is recognized at the inside by CitP and extruded again in exchange for citrate. While a substrate that is translocated from out-to-in is necessarily also translocated in the reverse direction, the affinity for the substrate at the two sides of the membrane may be very different. Best example would be the affinity of CitP for lactate which is in the milimolar range at the outside (3) and the micromolar range at the

cytoplasmic side of the membrane (Fig 6C) (18). Similarly, substrates like acetate, propionate, glyoxylate, and succinate that were readily identified as substrates of CitP in the citrate consumption assay (Fig. 3A, B, and 7A, respectively), were not identified as substrates in the transport assays, most likely because of too low an affinity of the externally facing binding site.

Substrates of CitP are identified when the initial rate of citrate consumption is higher in the presence than in the absence of the substrate. The latter condition represents the symport mode of uptake of citrate by CitP that is rate limited by the isomerization of the 'empty' binding site. The observed initial rate reflects the recycling rate of the substrate over the membrane that is determined by the activity of CitP and the influx into the cell by passive diffusion or the action of a transporter other than CitP (Fig. 2B and C). Passive permeation depends on hydrophobicity of the molecule because it has to pass the hydrophobic interior of the lipid membrane and the pK of the carboxylate group(s) because it determines the fraction in the permeative protonated form. In addition, the size of the side chain may play a role. The hydrophobicity of the R groups of the amino acid analog monocarboxylate substrates used in this study is high (Table S1) and it may be expected that the citrate consumption rate is determined by the activity of CitP. Therefore, the preference of CitP for OH>O>H groups at the C2 atom in case of f.i. the analogs of leucine, appears to be a justified conclusion. Dicarboxylates are more hydrophilic by nature, but the pK's are higher, and therefore, they are not less permeative *a priori*. The pK values together with an increasing hydrophobicity with chain length suggest an increase in membrane permeability in the order oxalate (C2) < malonate (C3) < succinate (C4) < glutarate (C5) < adipate (C6) (Table S2). The latter three accelerated the citrate consumption rate indicating rapid entry into the cell and recognition by CitP. Of the three C4-dicarboxylates, succinate and maleate entered the cell and were exchanged with citrate, while fumarate was not. Since maleate is the probably least permeative (lowest pK_1; Table S2), most likely a transporter is responsible for the uptake of the former two. So far, a known C4-dicarboxylate transporter has not been identified in the genome of *L. lactis* IL1403 (5).

In principle, the citrate consumption assay identifies substrates at the external face of the membrane as well, since they compete with external citrate. For most substrates, this is not observed since citrate, a high affinity substrate, is present in excess and/or the acceleration of the rate by internal substrate is dominant. In the absence of the latter, f.i. when the substrate is not permeative, high concentrations of the dicarboxylates, i.e. oxalate, malonate, malate, oxaloacetate, and fumarate were shown to inhibit the consumption rate to some extent, demonstrating that CitP has affinity for these compounds as well.

Substrate recognition by CitP

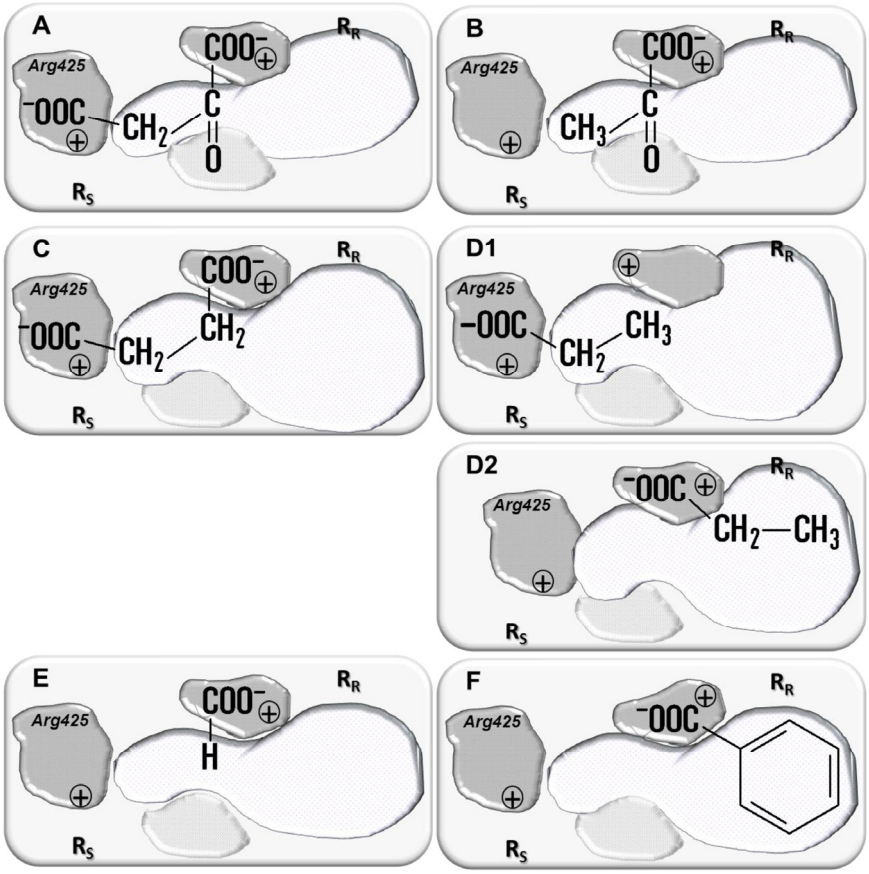
The substrate binding pocket of CitP contains sites that specifically interact with a substrate (Fig. 1). The interactions contribute to the affinity of the protein for the substrate and affect the transition between the inside and outside facing binding sites of the transporter. The latter is slow

in the absence of a bound substrate and fast when a substrate is bound, which is the basis of the assay used to identify substrates of the protein (see above). In view of the broad substrate specificity of CitP, not all interactions have to be satisfied. The interaction with a single carboxylate group on the substrate is both essential and sufficient (formate) (Fig. 6A and 8E). Interaction of the Arg425 residue in the R_S site with a second carboxylate group on the substrate is not essential which allows the transporter to exchange mono and divalent substrates, f.i. $Hcit^{2-}$/ lac^- exchange, which is the basis of membrane potential generation (Fig. 1). The interaction with the 2-hydroxyl group is also not essential, but results in better overall interaction when compared to a 2-keto group or the acids. With a 2-keto group, the nature of the interaction may be the same and monocarboxylate 2-keto acids would be oriented in the pocket in the same way as the 2-hydroxy acids (Fig. 1 and 8A, B). Replacement of the hydroxy group with H results in loss of the interaction and possibly monocarboxylates have two binding modes in which the carboxylate interacts with either of the carboxylate-binding site on the protein depending on the R group (Fig. 8D1, D2). Remarkably, the preference of CitP for the keto or acid analogs of X-CHR-COO^- (X is O or H, respectively) appears to depend on the bulkiness of side chain R. The acid is preferred for the glycine (X=NH_3^+) analogs, no preference is observed for alanine and valine analogs, while the keto acids are preferred for the leucine, phenylglycine, and phenylalanine analogs (Fig. 3 and Table S1). Dicarboxylates like succinate are likely to interact with both carboxylate binding sites on the protein (Fig. 8C). Finally, the different interactions between substrate and protein in the binding pocket seem to have different contributions to affinity for the substrate and lowering of the activation energy of the isomerization transition. Formate that only interacts with a carboxylate binding site on the protein only poorly stimulates the isomerization, but binds with relative high affinity (Fig. 6A and 8E). It may be noted that the binding pocket with bound formate, the smallest possible substrate, is most reminiscent of the 'empty' pocket that shows the slowest isomerization. In contrast, the affinity for benzoic acid is considerably lower, but the isomerization is as fast as observed for lactate (Fig. 6B, C). Apparently, the interaction with the hydrophobic phenyl group stimulates the isomerization, but negatively affects affinity (Fig. 8F).

Physiology of CitP substrates

The citrate transporter CitP transports a broad range of mono- and dicarboxylates that are found in the metabolome of LAB and that play an important role in flavor perception of dairy products (23). Those compounds are used in food industry as flavor enhancers or food additives (20) (see Table S1 and S2). Products of citrate and sugar fermentation and lipolysis like acetate and propionate, give a pungent, sour milk flavor and, products of amino acids metabolism like isovalerate or phenylacetate, are important in flavor and taste of cheddar cheese (6). The first step in degradative amino acid pathways is transamination yielding the corresponding keto acid, f.i. α-ketoisocaproate from leucine, α-ketoisovalerate from valine, phenylpyruvate from phenylalanine, and 2-keto-4-methylthiobutyrate from methionine. Formation of keto acids from

Figure 8. Substrate binding modes of CitP.



Substrates depicted in the binding pocket are oxaloacetate (A), pyruvate (B), succinate (C), propionate (D1 and D2), formate (E), and benzoate (F). For further explanation see the legend to Fig. 1 and the text.

branched chain amino acids, aromatic amino acids, and methionine in *L. lactis* IL1403 is catalyzed by two transaminases BcaT and AraT. The keto acids produced in this way may be reduced to the corresponding hydroxy acids by activity of 2-hydroxy acid dehydrogenases (HA-DH) at the expense of NADH. Genome analysis of *L. lactis* IL1403 has revealed a potential HA-DH encoding gene, L-2-hydroxyisocaproate dehydrogenase *hicD* (5, 23) (see Table S1). All these compounds were shown to be substrates of CitP in this study and are flavor compounds or precursors thereof. Furthermore, naturally occurring compounds that play an important role in plant growth as auxins, i.e. phenylacetate (PAA) and indole-3-lactate (ILA), the precursor of indole-3-acetate (IAA), are produced by metabolism of tryptophan in soil bacteria (1). IAA can be produced from indole-3-pyruvate (IPA, α-keto acid of Trp) by oxidative decarboxylation initiated by the decarboxylase IPA-DC encoded in the genome of *L. lactis* IL1403 (5, 23). Possibly, CitP plays a role in exporting these compounds out of the cell and has a much broader physiological function than uptake of citrate in the citrate fermentation pathway alone. The encoding of CitP on an endogenous plasmid while the metabolic enzymes are chromosomally encoded in *L. lactis* strains supports this view.

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Chapter 5

Rerouting citrate metabolism in *Lactococcus lactis*: citrate driven transamination

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Abstract

Oxaloacetate is an intermediate of the citrate fermentation pathway that accumulates in the cytoplasm of *Lactococcus lactis* ILCitM(pFL3) at high concentration due to the inactivation of oxaloacetate decarboxylase. Excess of toxic oxaloacetate is excreted into the medium in exchange with citrate by the citrate transporter CitP (A. M. Pudlik and J. S. Lolkema, *J. Bacteriol.* 193:4049-56, 2011). In this study, transamination of amino acids with oxaloacetate as the keto donor is described as an additional mechanism to relieve toxic stress. Redirection of the citrate metabolic pathway into the transamination route in the presence of the branched-chain amino acids Ile, Leu, and Val, the aromatic amino acids Phe, Trp, and Tyr, and Met resulted in the formation of aspartate and the corresponding α-keto acids. Cells grown in the presence of citrate showed 3.5-7 times higher transaminase activity in the cytoplasm than cells grown in the absence of citrate. The study demonstrates that transaminases of *L. lactis* accept oxaloacetate as keto donor. A significant fraction of 2-keto-4-methylthiobutyrate formed from methionine by citrate driven transamination *in vivo* was further metabolized yielding the cheese aroma compounds 2-hydroxy-4-methylthiobutyrate and 3-methylthiopropionate. Reducing equivalents required for the former compound were produced in the citrate fermentation pathway as NADH. Similarly, phenylpyruvate, the transamination product of phenylalanine, was reduced to phenyllactate, while the dehydrogenase activity was not observed for the branched-chain keto acids. Both α-keto acids and α-hydroxy acids are known substrates of CitP and may be excreted from the cell in exchange with citrate or oxaloacetate.

Introduction

Transamination of amino acids is a key step in the formation of aroma compounds by Lactic Acid Bacteria (LAB) in the food fermentation industry (24, 30). The reaction is a first step in amino acids catabolism and is catalyzed by pyridoxal-5′-phosphate (PLP) dependent aminotransferases that convert amino acids into the corresponding α-keto acids. At the same time, a keto donor, usually α -ketoglutarate, is converted to the corresponding amino acid (i.e. glutamate). The α-ketoacids are the precursors of many flavor compounds, like aldehydes, alcohols, carboxylic acids, or α-hydroxy acids that are formed by subsequent enzymatic or spontaneous reactions, like decarboxylation, dehydrogenation, or oxidation. Unfortunately, for the dairy industry, conversion of amino acids into aroma compounds by LAB is often limited by lack of sufficient keto donor (27).

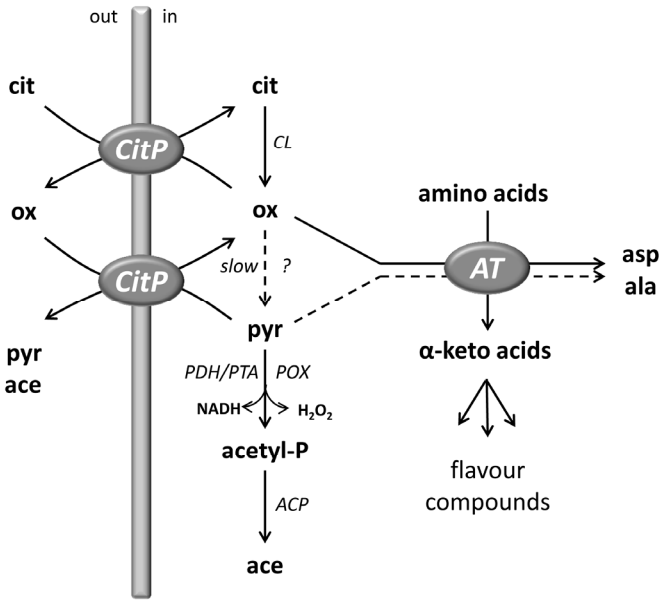
Citrate constitutes almost 90 % of organic acids in milk, where it is present at concentrations ranging from 4-10 mM (19). Citrate fermentation by LAB leads to the production of two potential keto donors for transamination reactions, i.e. oxaloacetate and pyruvate (8). However, the two best characterized transaminases from LAB, AraT, specific for aromatic amino acids (20), and BcaT, specific for branched-chain amino acids (29), were reported to accept neither oxaloacetate nor pyruvate as keto donor in their purified forms (20, 28). The aspartate transaminase AspC encoded on the genome of *L. lactis* IL1403 by the *aspB* gene (2) did accept oxaloacetate (4), while the closest homolog of the alanine transaminase AlaT (encoded by the *aspC* gene) (2) found in *Corynebacterium glutamicum* showed the highest activity with pyruvate as keto donor (12, 14). Moreover, analysis of *L. lactis* IL1403 genome sequence (2) revealed the presence of 9 other putative transaminase genes among which *ytjE* that was reported to be involved in cysteine and methionine metabolism (13). None of the other enzymes was functionally characterized.

Recently, accumulation of oxaloacetate in the cells of *Lactococcus lactis*, which is widely used as a starter in the dairy industry, was demonstrated (17). In resting cells, citrate fermentation in the oxaloacetate decarboxylase deficient mutant strain *L. lactis* ILCitM(pFL3), a derivative of strain IL1403 (1), was shown to proceed in two steps (Fig. 1) (17). The first step consists of a short pathway in which two enzymes are involved: citrate transporter CitP and citrate lyase (CL). CL converts internal citrate into oxaloacetate (and acetate) with high efficiency. Oxaloacetate is rapidly accumulating in the cytoplasm and excreted in exchange with citrate by CitP. In the second step that follows upon complete consumption of citrate, oxaloacetate reenters the cell via CitP in exchange with available intermediates/end products pyruvate (pyr) and/or acetate (ace). During the whole process, internal oxaloacetate is slowly converted into pyruvate by the activity of a cryptic decarboxylase. Under these conditions, pyruvate from citrate is converted to the end product acetate (17).

The present study demonstrates that oxaloacetate produced by the oxaloacetate deficient mutant strain *L. lactis* ILCitM(pFL3) can be used efficiently to drive transamination in the presence of amino acids. The α-keto acids produced from the amino acids are either exported by CitP into the medium (18) or further metabolized into flavor compounds. The cells use the transamination

pathway as a second escape route to relieve the stress caused by the toxic levels of oxaloacetate in addition to excretion of the intermediate out of the cell.

Figure 1. Citrate driven transamination in *L. lactis* ILCitM(pFL3).



See text for explanation. CitP, citrate transporter; CL, citrate lyase; ?, cryptic oxaloacetate decarboxylase; PDH, pyruvate dehydrogenase complex; PTA, phosphotransacetylase; POX, pyruvate oxidase; ACP, acylphosphate phosphohydrolase; AT, aminotransferase. cit, citrate; ox, oxaloacetate; pyr, pyruvate; acetyl-P, acetyl phosphate; ace, acetate; asp, aspartate; ala, alanine.

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Materials and methods

Chemicals

2-hydroxy-4-methylthiobutyrate, 2-keto-4-methylthiobutyrate, acetate, aldehyde dehydrogenase, citrate, diethylethoxymethylenemalonate (DEEMM), L-alanine, L-aminoadipic acid, L-cysteine, L-glutamine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, L-valine, methyl-3-methylthiopropionate, oxaloacetate, phenyllactate, phenylpyruvate, pyridoxal 5′-phosphate (PLP), pyruvate, α-ketoglutarate, α-ketomethylvalerate, α-ketoisocaproate, and α-ketoisovalerate were obtained from Sigma Aldrich Chemicals. L-arginine, L-asparagine, L-aspartate, L-glutamate, L-glycine, and L-methionine were obtained from Merck. Solutions of amino acids were prepared in 50 mM potassium phosphate pH 5.8 buffer; if required, the pH was adjusted to 5.8 with 5 M KOH. L-lactate dehydrogenase (L-LDH), L-malate dehydrogenase (L-MDH), and citrate lyase (CL) were obtained from Roche Applied Science. 3-methylthiopropionic acid was obtained by saponification of methyl-3-methylthiopropionate (25) or by enzymatic reaction of methional catalyzed by 1U of aldehyde dehydrogenase in the presence of 1.3 mM NAD^+ at pH 8.0 at 37 ^oC (26).

Bacterial strains and growth conditions

Strain *Lactococcus lactis* IL1403(pFL3) (11) and the oxaloacetate decarboxylase deficient derivative ILCitM(pFL3) (1) were used in this study. Plasmid pFL3 harbors the lactococcal CRL264 *citP* gene under control of the *Streptococcus pneumoniae polA* promoter (11). Neither expression nor plasmid copy number is under control of citrate or pH in these strains (6). Mutant strain ILCitM(pFL3) was constructed by a deletion of 14 bp between positions 584 and 598 of the oxaloacetate decarboxylase gene encoded by the *mae* gene (1). Precultures were grown overnight at 30 ^{o}C in M17 broth medium supplemented with 0.5 % (w/v) glucose (M17G) and 5 μg.ml^{-1} of tetracycline. Cells were grown in M17G medium at an initial pH adjusted to 7.0. When indicated, 20 mM of citrate pH 7.0 was added to the medium. Growth was performed in 100 ml serum bottles without agitation and at 30 ^{o}C. Growth was followed by measuring the optical density at a wavelength of 660 nm. Cells were harvested at mid-exponential growth phase when the optical density was 0.6 by spinning for 10 min at 3000 rpm. Cells were washed two times with 50 mM potassium phosphate pH 5.8 buffer, and, finally, resuspended in the same buffer at 4 ^{o}C.

Citrate, oxaloacetate, or pyruvate driven transamination

Resting cells at an OD_{660} of 1.5 in 50 mM potassium phosphate pH 5.8 buffer were incubated at 30 ^{o}C without agitation for 10 min. The assay was performed in a total volume of 1.5 ml. At t=0, 2 mM of citrate, oxaloacetate, or pyruvate was added in the presence or absence of 2 mM of amino acid and 50 μM of PLP. Samples of 50-100 μl were taken at the indicated times and immediately centrifuged for 0.5 min at maximum speed in a table top centrifuge. The supernatant was stored on ice or frozen until further analysis by enzymatic assays and/or HPLC.

Enzymatic assays

Citrate, oxaloacetate, and pyruvate were measured as described before (16), using the commercially available enzymes citrate lyase (CL), L-malate dehydrogenase (L-MDH), and L-lactate dehydrogenase (L-LDH). Briefly, an aliquot of 30 μl of the sample was added to 50 mM TrisHCl pH 7.8 buffer containing NADH. Oxaloacetate in the sample was converted to L-malate at the expense of NADH after addition of L-MDH. Pyruvate in the sample was measured by addition of L-LDH which results in the conversion of pyruvate to L-lactate at the expense of NADH. Addition of CL converts citrate in the sample to oxaloacetate (and pyruvate) resulting in an additional decrease in the NADH concentration equivalent to the citrate concentration present in the sample. The assay was performed in 96-well microtiter plates. The decrease in NADH concentration was measured spectroscopically at 340 nm. Standard deviations were obtained from at least 3 different experiments.

HPLC/RP-HPLC analysis

Samples were run on a Shimadzu high-speed HPLC Prominence UFLC and later analyzed using

the LC Solutions 1.24 SP1 software from Shimadzu (Kyoto, Japan). Products of citrate metabolism and transamination (α-keto acids and further metabolites) were determined by loading an aliquot of 10 µl of the supernatant on an Aminex HPX-87H anion-exchange column with dimensions 300 x 7.8 mm (Bio-Rad Laboratories, Inc., Richmond, CA) operated at 30 ^{o}C in isocratic mode using 0.005 M H_2SO_4 as the mobile phase and a flow rate of 0.8 ml/min.

Amino acids were analyzed by RP-HPLC after DEEMM derivatization. Aminoenone derivatives of amino acids were obtained by reaction of 175 µl of 1 M borate pH 9.0 buffer, 75 µl of methanol, 2 µl of 0.1 % (w/v) of L-aminoadipic acid, 3 µl of DEEMM, and 100 µl of supernatant in a 1.5 ml closed tube over 30 min incubation at room temperature in an ultrasound bath. Then, the sample was incubated at 70 ^{o}C for 2 h to allow complete degradation of the excess of DEEMM (7). Detection of aminoenone derivatives was performed in an Alltech Platinum EPS C18 column with dimensions 250 x 4.6 mm operated at 25 ^{o}C through the binary gradient shown in Table 1 with a flow rate 0.8 ml/min. Eluent A was 25 mM acetate pH 5.8 buffer with 0.02 % sodium azide and eluent B was 80:20 mixture of acetonitrile and methanol. The target compounds were identified according to the retention times and were quantified using the external standard method. Measurements of the concentrations of citrate, oxaloacetate, and pyruvate were in good agreement between the enzymatic and HPLC methods. Standard deviations were obtained from at least 3 different experiments.

Table 1. Eluent gradient for HPLC determination of aminoenone derivatives of amino acids.

Time (min)	0	13	13.5	17	20	32	40	45	48	50
Eluent A (%)	94	84	82	82	78	68	5	5	94	94
Eluent B (%)	6	16	18	18	22	32	95	95	6	6

Results

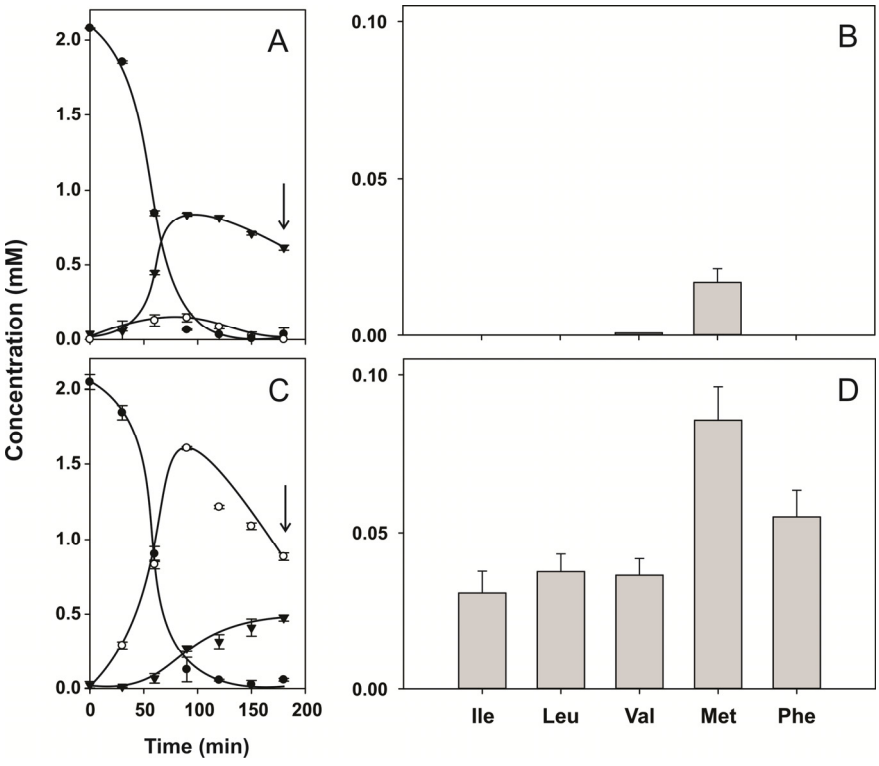
Citrate driven transamination

Lactococcus lactis strain IL1403(pFL3) harbors plasmid pFL3 that encodes the citrate transporter CitP under control of a constitutive promoter (see Materials and methods). Together with the metabolic enzymes encoded on the chromosome, the transporter completes the citrate fermentation pathway. The citrate fermentation pathway in *L. lactis* IL1403 produces two potential keto donors for transamination activity, i.e. oxaloacetate and pyruvate (Fig. 1) (16). Resting cells of *L. lactis* IL1403(pFL3) were incubated in 50 mM KPi pH 5.8 containing 50 µM of the cofactor PLP in the presence of 2 mM of citrate, and 2 mM of the amino acids Ile, Leu, Val, Met, or Phe. The highest concentrations of pyruvate and oxaloacetate produced by the cells, 0.85 and 0.15 mM, respectively, were observed when all citrate was consumed after 90 minutes. The remainder of citrate added was converted to acetate (not shown) (16). Following depletion of citrate,

pyruvate, and oxaloacetate were taken up and metabolized by the cells again (16, 17). After another 90 min, when pyruvate was present at a concentration of 0.6 mM and no oxaloacetate was detected anymore (Fig. 2A, arrow), measurement of the corresponding α-keto acids (see Fig. 1) of Ile, Leu, Val, Met, or Phe by HPLC revealed very little transamination activity by the cells (Fig. 2B). Only in the presence of methionine, 20 μM of 2-keto-4-methylthiobutyrate was detected. Control experiments showed that the α-keto acid of methionine was formed only when both citrate and methionine were present.

L. lactis strain ILCitM(pFL3), a derivative of strain IL1403(pFL3), is deficient in oxaloacetate decarboxylase (1). Consequently, incubation in the presence of 2 mM citrate and one of the five amino acids resulted in the production of 1.6 mM oxaloacetate after complete consumption of citrate that was a bit slower than in the parent strain (Fig. 2C) (17). Subsequently, oxaloacetate was consumed again to reach a concentration of 0.9 mM after 3 h. The pyruvate concentration slowly increased during the incubation period to reach a concentration of 0.5 mM. In contrast to the parent strain, the oxaloacetate decarboxylase deficient strain produced significant amounts of the α-keto acids of all five amino acids (Fig. 2D). The highest production was observed from Met

Figure 2. Citrate metabolism (A, C) and α-keto acid production (B, D) by resting cells of *L. lactis* IL1403(pFL3) (A, B) and ILCitM(pFL3) (C, D).



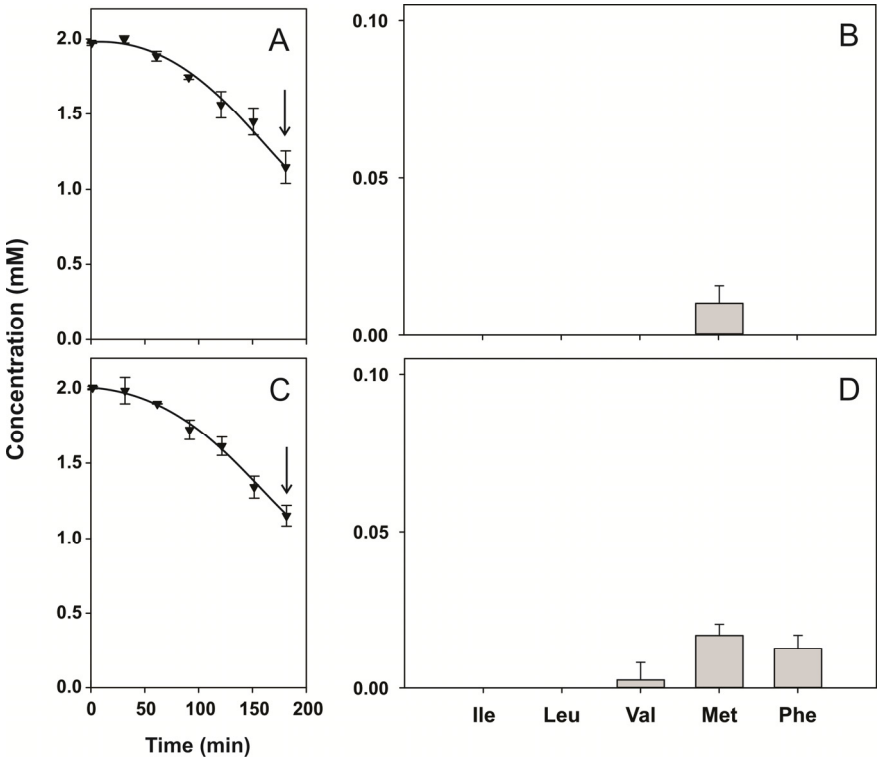
Resting cells were incubated with 2 mM of citrate and 2 mM of the amino acids: Ile, Leu, Val, Met, and Phe. A, C. (●) citrate, (○) oxaloacetate, (▼) pyruvate. B, D. The corresponding α-keto acids of Ile, Leu, Val, Met, and Phe were determined after 3 h of incubation.

(80-90 μM) followed by Phe (50-60 μM) and the branched-chain amino acids (30-40 μM). In all cases, transamination was strictly dependent on the presence of both citrate and the amino acids, while the production of the α-keto acids was strongly reduced when the cofactor PLP was omitted from the buffers (not shown). Hence, measured α-keto acids are products of PLP-depended amino acid transamination driven by metabolism of citrate in the mutant strain. The kinetics of citrate consumption and oxaloacetate and pyruvate formation was not significantly affected by the presence or absence of the amino acids in the mutant strain indicating that the flux going into the transamination route was minor.

Oxaloacetate and pyruvate driven transamination

The higher concentration of oxaloacetate and lower concentration of pyruvate produced from citrate by the mutant strain ILCitM(pFL3) suggested that the former is the keto donor in the transamination reactions. In agreement, incubation of both the parent and mutant strains in the presence of 2 mM of pyruvate and either one of the five amino acids did not result in the amounts of α-keto acids that were observed with the mutant strain in the presence of citrate (Fig. 3B, D and Fig. 2B, D, respectively). In fact, for both strains, the result was very similar as observed for the

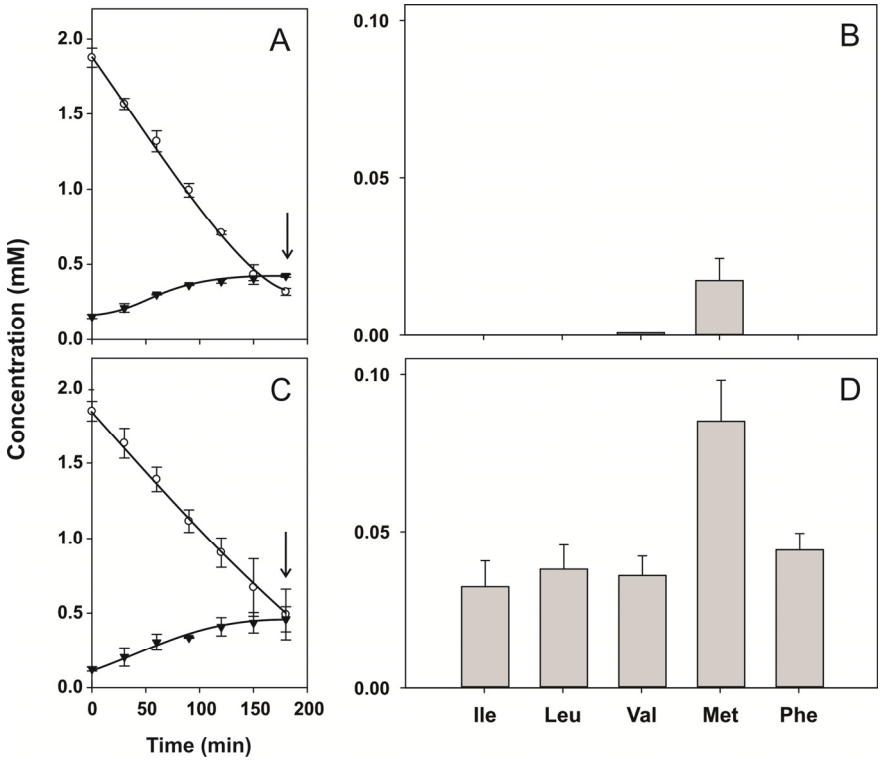
Figure 3. Pyruvate metabolism (A, C) and α-keto acid production (B, D) by resting cells of *L. lactis* IL1403(pFL3) (A, B) and ILCitM(pFL3) (C, D).



Resting cells were incubated with 2 mM of pyruvate and 2 mM of the amino acids: Ile, Leu, Val, Met, and Phe. A, C. (▼) pyruvate. B, D. The corresponding α-keto acids of Ile, Leu, Val, Met, and Phe were determined after 3 h of incubation.

parent strain in the presence of citrate (Fig. 3). When after 3 h of incubation, 0.85 mM of pyruvate was consumed (Fig. 3A, C), 10-20 μM of the α-keto acid of methionine was produced (Fig. 3B, D). In addition, the mutant strain produced 15 μM of phenylpyruvate, the α-keto acid of Phe, while no α-keto acids formed from the branched-chain amino acids could be detected (Fig. 3D). Apparently, pyruvate is a poor keto donor for transaminases of *L. lactis* IL1403. In contrast, incubation of the mutant and parent strains in the presence of oxaloacetate and any of the five amino acids resulted in the same pattern of α-keto acid production as observed in the presence of citrate (Fig. 4 and 2). Oxaloacetate is taken up by the cells by the citrate transporter CitP (see Fig. 1) (17). In the parent strain, internalized oxaloacetate is rapidly decarboxylated giving low cytoplasmic oxaloacetate concentrations (16). In agreement with the above results, the decarboxylation product pyruvate does not enter the transamination pathway efficiently (Fig. 4A, B). In the oxaloacetate decarboxylase deficient strain, the oxaloacetate concentration in the cytoplasm will be much higher, supporting significant transamination (Fig. 4C, D). Remarkably, in spite of the different kinetics of cytoplasmic oxaloacetate conversion, the overall kinetics of oxaloacetate consumption and pyruvate excretion was quite similar in the two strains.

Figure 4. Oxaloacetate metabolism (A, C) and α-keto acid production (B, D) by resting cells of *L. lactis* IL1403(pFL3) (A, B) and ILCitM(pFL3) (C, D).

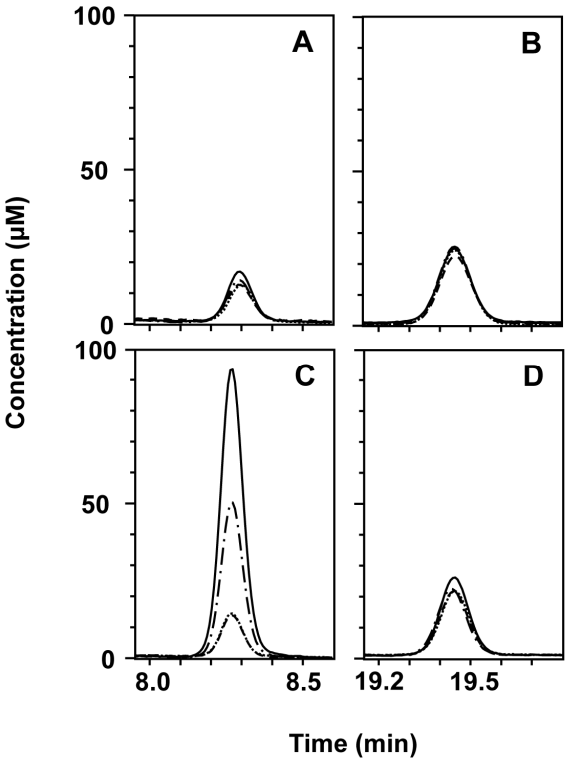


Resting cells were incubated with 2 mM of oxaloacetate and 2 mM of the amino acids: Ile, Leu, Val, Met, and Phe. A, C. (○) oxaloacetate, (▼) pyruvate. B, D. The corresponding α-keto acids of Ile, Leu, Val, Met, and Phe were determined after 3 h of incubation.

Production of aspartate and alanine by ILCitM(pFL3)

In the transamination reaction, keto donors oxaloacetate and pyruvate result in the formation of the amino acids aspartate and alanine, respectively (Fig. 1). Production of amino acids by the cells under the same conditions as described above was measured by RP-HPLC as described in the Materials and methods. After 3 h of incubation and in the absence of any further additions, resting cells of both parent and mutant strains produced 15 µM of aspartate and 25 µM of alanine (Fig. 5). Most likely, some proteolytic activity resulted in the release of free amino acids from the cells. No significant differences were observed when the parent strain was incubated in the presence of 2 mM citrate or 2 mM isoleucine or both (Fig. 5A, B), in line with the lack of formation of α-ketomethylvalerate, the α-keto acid formed from isoleucine, described above (Fig. 2B). Similarly, the alanine concentration was not affected when the mutant strain was incubated in the presence of these additions, in line with the lack of pyruvate driven transamination (Fig. 5D). In contrast, the aspartate concentration was the same in the presence of 2 mM isoleucine, but raised up to 50 µM in the presence of 2 mM citrate and to 95 µM in the presence of both isoleucine and citrate (Fig. 5C). Straightforward explanation for the increase in the presence of citrate alone would be transamination between oxaloacetate and amino acids produced in the cell by proteolytic activity. The additional increase in the presence of isoleucine demonstrates that α-ketomethylvalerate produced by the mutant strain is the result of oxaloacetate:isoleucine transaminase activity. The experiments were repeated with the other amino acids Leu, Val, Phe, and Met (Table 2). In all cases, aspartate was produced without significant amounts of alanine

Figure 5. Aspartate (A, C) and alanine (B, D) production during citrate driven transamination by resting cells of *L. lactis* IL1403(pFL3) (A, B) and *L. lactis* ILCitM(pFL3) (C, D).



Resting cells were incubated for 3 h without further additions (– – –), in the presence of 2 mM citrate (• — • — •), 2 mM Ile (--------), and 2 mM citrate and 2 mM Ile (———). The graphs represent the RP-HPLC elution profile.

Table 2. Transamination of 5 natural amino acids in *L. lactis* ILCitM(pFL3).

aa[a]	Asp (µM)[b]	Ala (µM)[b]
Met	124 ± 13	13 ± 10
Phe	70 ± 20	5 ± 3
Val	56 ± 13	8 ± 7
Ile	50 ± 6	6 ± 4
Leu	51 ± 9	7 ± 3

[a] amino acids were added at the concentration of 2 mM in the presence of 2 mM citrate and 50 µM of PLP;

[b] amount of Asp and Ala were measured after 3 h of citrate driven transamination; amount of Asp and Ala produced from citrate in the absence of added amino acids was subtracted.

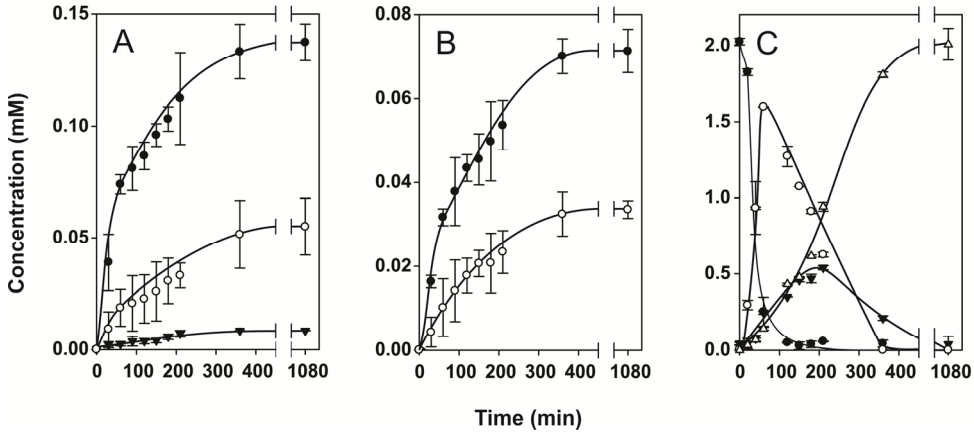
demonstrating that oxaloacetate and not pyruvate is the keto group donor in citrate driven transamination. In addition to aspartate and alanine, low concentration of Glu, Asn, Gln, Gly, Thr, and Lys were detected after 3 h of incubation under each experimental condition supporting the proteolytic or autolytic activity of the cells.

Products of citrate driven transamination from methionine and phenylalanine

α-Keto acids are precursors of other flavor compounds (Fig. 1). Further metabolism of 2-keto-4-methylthiobutyrate, the α-keto acid of methionine, by resting cells of *L. lactis* ILCitM(pFL3) resulted in two additional products that were identified as the reduced form 2-hydroxy-4-methylthiobutyrate and 3-methylthiopropionic acid (MTPA) (Fig. 6A). Phenylpuruvate, the α-keto acid of phenylalanine, was converted to a single compound that was identified as the reduced form phenyllactate (Fig. 6B). The downstream products were only detected in the presence of both citrate and the amino acid. No reduced α-keto acid or any other compounds were produced in the presence of the branched-chain amino acids. Final concentrations of the oxidized and reduced forms produced from methionine were 140 and 50 µM, respectively, and from phenylalanine 70 and 35 µM, respectively (Fig. 6A, B) which is in good agreement with the amounts of aspartate formed (Table 2). Together with the relatively modest production of 10 µM of MTPA, it follows that, under the conditions of the experiment, in the presence of methionine, 10 % of citrate was directed into the transamination route.

The production of the oxidized and reduced forms from methionine and phenylalanine followed similar kinetics that appeared to be linked directly to the kinetics of citrate metabolism (Fig. 6C). The rate was highest during the first 60-70 min when citrate was consumed and external oxaloacetate reached its maximum (Fig. 6A, B, and C). During the reuptake and consumption of oxaloacetate, the production proceeded at a slower pace to more or less come to a halt when all oxaloacetate was consumed after approximately 6 h. Remarkably, following the depletion of oxaloacetate the oxidized forms were not further converted into the reduced forms, strongly suggesting that the required reducing equivalents were produced in the citrate metabolic pathway (see Discussion).

Figure 6. Formation of transamination products of methionine (A) and phenylalanine (B).



Further metabolism of 2-keto-4-methylthiobutyrate (α-keto acid of methionine) and phenylpyruvate (α-keto acid of phenylalanine) by citrate metabolism (C) in resting cells of *L. lactis* ILCitM(pFL3). Resting cells were incubated in the presence of 2 mM citrate and 2 mM of Met (A, C) and Phe (B). A. (●) 2-keto-4-methylthiobutyrate, (○) 2-hydroxy-4-methylthiobutyrate, (▼) 3-methylthiopropionic acid (MTPA). B. (●) phenylpyruvate, (○) phenyllactate. C. (●) citrate, (○) oxaloacetate, (▼) pyruvate, (Δ) acetate (formed in addition to acetate formed by citrate lyase).

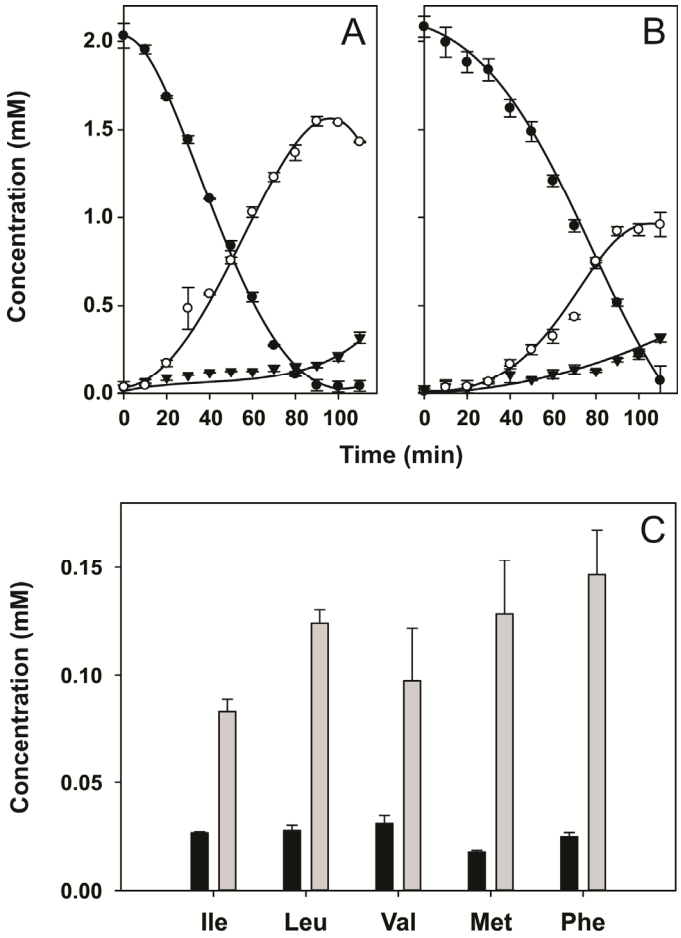
5

Oxaloacetate stress response

The strains of *L. lactis* used in the studies thus far were grown in the absence of citrate to prevent oxaloacetate stress in the mutant strain during growth (1, 17). Rerouting the citrate fermentation pathway into transamination may release the stress condition. The kinetics of citrate consumption and oxaloacetate production by resting cells of *L. lactis* ILCitM(pFL3) grown in the presence of 20 mM citrate was a little, but significantly slower than observed in the presence of citrate (Fig. 7A, B). The highest concentration of oxaloacetate measured outside the cells grown in the presence of citrate was about 40 % lower, 0.95 *versus* 1.5 mM, while the amount of pyruvate produced did not differ significantly. In spite of the lower oxaloacetate concentration produced by the stressed cells, citrate driven transamination in the presence of the five amino acids was only marginally different than observed with the cells grown in the absence of citrate (not shown). Besides transamination activity in the cytoplasm, transamination by the cells involves other steps such as citrate lyase activity, uptake of citrate and the amino acid, and excretion of aspartate and the α-keto acid product. To isolate the transamination capacity in the cytoplasm from the process as a whole, cells were permeabilized with 0.15 % Triton X-100 for 20 min at 30 ^{o}C to omit the transport steps and oxaloacetate was used as the keto donor to bypass citrate lyase. The permeabilized cells of the mutant strain grown in the absence of citrate produced approximately 25 % less of the corresponding α-keto acids from the three branched-chain amino acids (compare Fig. 7C and 4D). The production from phenylalanine and, especially, methionine was significantly more reduced which at least in part may be explained by the lack of the formation of the reduced form of the α-keto acids but an inhibitory effect of the Triton X-100 treatment also should not be

excluded. Assuming that the activities in the two types of permeabilized cells were comparable, the results indicated that the cytoplasmic transamination activity with the branched-chain amino acids was on average 3.5 times higher in the stressed cells. Similarly, the activity with methionine and phenylalanine was found to be about 7 times higher in the stressed cells (Fig. 7C). It follows that oxaloacetate stress sensed by the cells results in an increase of transamination activity, most likely by an up regulation of the expression of the involved transaminases.

Figure 7. Oxaloacetate stress response in *L. lactis* ILCitM(pFL3).



A, B. Resting cells of *L. lactis* ILCitM (pFL3) grown in the absence (A) and presence (B) of 20 mM of citrate were incubated with 2 mM citrate and 2 mM of the amino acids: Ile, Leu, Val, Met, and Phe. (●) citrate, (○) oxaloacetate, (▼) pyruvate. C. The corresponding α-keto acids of Ile, Leu, Val, Met, and Phe were determined after 3 h of incubation of cells grown in the absence (filled bars) or presence (grayed bars) of 20 mM citrate and treated with 0.15 % Triton X-100 for 20 min before addition of 2 mM oxaloacetate and 2 mM of the amino acids.

Discussion

Coupling citrate fermentation and amino acids catabolism through transamination

The aim of the present study was to reroute citrate metabolism in *L. lactis* into the transamination route to boost amino acids catabolism. Breakdown of amino acids is an important first step in the

production of flavor compounds in the cheese industry, but often limited by the availability of a keto donor, while amino acids are in access in the protein rich cheese matrix (24, 30). Citrate that is present in relatively high amounts in milk (19) is split by citrate lyase into acetate and oxaloacetate, a potential keto donor in the transamination reaction. Oxaloacetate was shown to accumulate to high concentrations in the cytoplasm when the next step in the pathway was blocked by inactivation of oxaloacetate decarboxylase in strain *L. lactis* ILCitM(pFL3) (1, 17). Results presented here, clearly show that the mutant strain, but not the parent strain, produced significant amounts of the α-keto acids α-ketomethylvalerate, α-ketoisocaproate, α-ketoisovalerate, phenylpyruvate, and 2-keto-4-methylthiobutyrate from the branched-chain amino acids isoleucine, leucine, valine, the aromatic amino acid phenylalanine, and the sulfur containing amino acid methionine, respectively. The keto donor in the transamination reaction was shown to be oxaloacetate, and not pyruvate, by the same α-keto acid product profiles obtained when the parent and mutant strains were incubated with oxaloacetate rather than citrate (see Fig. 2 and 4). In addition, aspartate, the product of oxaloacetate in the transamination reaction was formed and not alanine, the corresponding amino acid of pyruvate (Fig. 5). Clearly, in the mutant strain part of oxaloacetate derived from citrate is redirected into the transamination route. In the parent strain the steady state concentration in the cytoplasm is too low because of the high activity of the oxaloacetate decarboxylase (16, 17). The fraction of the citrate metabolic flux going into the transamination route is modest, at most 10 % in the case of methionine. However, in terms of flavor compounds production this is highly significant. A yield of 200 μM of flavor compounds within 6 h from methionine (Fig. 6A) that is one of the most important precursors of flavors in cheese manufacturing is much higher than observed in similar studies using α-ketoglutarate as the keto donor (20, 21).

Oxaloacetate stress response

The oxaloacetate deficient strain *L. lactis* ILCitM(pFL3) mimics the physiological condition where oxaloacetate accumulates in the cytoplasm. High concentrations of oxaloacetate are toxic to the cell and result in reduced growth rates and lower biomass yield (1, 17). Possibly, the high concentrations competitively inhibit other metabolic enzymes. Strains expressing the citrate transporter CitP, a very promiscuous carboxylate transporter (18), respond to the stress condition by the excretion of oxaloacetate into the medium in exchange with external citrate (substrate/product exchange), thereby reducing the cytoplasmic concentration (Fig. 1) (17). The present study uncovers a second response: the excess of internal oxaloacetate is funneled into the transamination pathway. The required amino acids will be available readily because of the high proteolytic activity of *L. lactis* (15). Stressed cells of strain ILCitM(pFL3) revealed an up regulation of transamination activity in the cytoplasm (Fig. 7C). In resting cell experiments, the increased transamination activity is obscured probably by limitations in the uptake and excretion of substrates and products, but in growing cells when energy sources are available and transport

reactions not limited, the effect may be substantial. For instance, if 10 % of the flux is directed to oxaloacetate:methionine transamination under unstressed conditions (Table 2), up regulation of the involved transaminase by a factor 7 (Fig. 7) may significantly contribute to the stress response. Then, the production of flavor compounds will be accordingly higher.

L. lactis transaminases

The genome sequence of *L. lactis* IL1403 contains 13 genes encoding putative transaminases (2). AraT is specific for aromatic amino acids (20), BcaT for branched-chain amino acids (29), AspC, the aspartate transaminase (encoded by *aspB*) (4), YtjE is involved in cysteine and methionine metabolism (13), and AlaT, the alanine transaminase (encoded by *aspC*) (14). Other genes annotated as encoding transaminases are *serC*, *argD*, *glmS*, *hisC*, *arcT*, *nifS*, *nifZ*, and *yeiG,* but none of these was functionally characterized in *L. lactis* (2). AraT and BcaT were previously reported to be major transaminases in cheese flavor development from aromatic amino acids and branched-chain amino acids, respectively (20, 21, 29). The purified enzymes showed activity with α-ketoglutarate as the keto donor and neither AraT nor BcaT showed activity with oxaloacetate or aspartate (20, 28). A double knock-out of *bcaT* and *araT* still showed transamination of aspartate (21) while inactivation of AspC resulted in no detectable activity with aspartate (4). Studies of the substrate specificity of BcaT, AraT, and AspC were done using α-ketoglutarate as the keto donor that is converted into glutamate. The activity of AspC with aspartate necessarily reveals that oxaloacetate is a substrate of this transaminase.

The present results show that oxaloacetate is an efficient keto donor in the transamination reactions catalyzed by *L. lactis* IL1403 for the amino acids Ile, Leu, Val, Phe, Met. In addition, of the 20 naturally occurring amino acids, the mutant cells were capable of producing aspartate from citrate in the presence of tyrosine, tryptophan, and glutamate (unpublished results). The substrate specificity of the oxaloacetate:amino acid transaminases overlap with the specificity of the α-ketoglutarate:amino acid transaminases BcaT and AraT. Either, the oxaloacetate specific transaminases are a separate set of enzymes or, more likely, BcaT and AraT in the cytoplasm of *L. lactis* have affinity for both keto donors.

Metabolic products of α-keto acids

α-Keto acids produced by aminotransferases are known precursors of flavor compounds converted by dehydrogenation to the corresponding α-hydroxy acids, decarboxylation to the corresponding aldehydes that are further converted into alcohols, carboxylic acids, and (thio)-esters, or dehydrogenation to the corresponding CoA-esters that are further metabolized to carboxylic acids. These compounds are produced in spontaneous or enzymatic reactions (24, 30). In the resting cell experiments presented here, only few of these downstream products showed up because of missing metabolic requirements, missing enzymes, or simply because they were not detected. A minor amount of 3-methylthiopropionic acid (MTPA) was formed from

methionine. MTPA adds a baked/boiled potato flavor to fermented food products and was identified as an important aroma note in cheddar cheese (23). It is formed directly by spontaneous oxidative decarboxylation of the α-keto acid that was reported before in other LAB (10). Most prominent downstream products observed were 2-hydroxy-4-methylthiobutyrate and phenyllactate formed from methionine and phenylalanine, respectively. α-Hydroxy acids are not major flavor compounds, but they play a role in flavor development in semi hard cheeses made with lactococci (24). The conversion of α-keto acids into α-hydroxy acids is catalyzed by hydroxyacid dehydrogenases (HA-DH) that are stereospecific enzymes with broad substrate specificity and widely distributed in LAB (5). The reduction of α-keto acids by resting cells of *L. lacti*s was previously observed after addition of glucose (22), however, no enzyme has been characterized. Genome analysis of *L. lactis* IL1403 revealed the putative HA-DH gene *hicD* annotated as L-2-hydroxyisocaproate dehydrogenase (2), however the enzyme has not been studied. More recently, PanE encoded by *panE* in *L. lactis* IL1403 was described as 2-hydroxyisocaproate dehydrogenase with the highest catalytic efficiencies for α-ketomethylvalerate, α-ketoisocaproate, and α-ketoisovalerate (3). However, in the present studies, no reduction of the branched-chain α-keto acids was observed. A better candidate would be L-lactate dehydrogenase encoded by *ldh* gene that was shown in *Lactobacillus plantarum* to be responsible for the reduction of α-keto acids derived from phenylalanine and methionine (3, 9).

The kinetics of production of the reduced and oxidized forms strongly suggested that the required reducing equivalents were produced in the citrate metabolic pathway. Under the conditions of the experiments, pyruvate from citrate is mainly converted to acetate via acetyl phosphate (16). Two operative routes from pyruvate to acetyl phosphate have been proposed: the NADH producing route catalyzed by pyruvate dehydrogenase complex (PDH) and phosphotransacetylase (PTA) and the hydrogen peroxide producing route catalyzed by pyruvate oxidase (POX) (see Fig. 1). The two routes are complemented to each other since H_2O_2 produced by POX is used to reoxidize NADH produced by PDH. During citrate driven transamination, reduction of α-keto acids into α-hydroxy acids at the expense of NADH produced by PDH may substitute H_2O_2 to control the NADH/NAD^+ balance in the cell.

Finally, the α-keto acids and especially α-hydroxy acids produced in the transamination reactions are substrates of the citrate transporter CitP (18). CitP may be central to a metabolic system of citrate driven transamination in which citrate is taken up in exchange with the excretion of the transamination products catalyzed by CitP.

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Chapter 6

Uptake of α-ketoglutarate by the citrate transporter CitP drives transamination in *Lactococcus lactis*

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6

Abstract

Transamination is the first step in the conversion of amino acids into aroma compounds by Lactic Acid Bacteria (LAB) used in food fermentations. The process is limited by the availability of α-ketoglutarate that is the best α-keto donor for transaminases in LAB. Here, uptake of α-ketoglutarate by the citrate transporter CitP is reported. Cells of *L. lactis* IL1403 expressing CitP showed significant levels of transamination activity in the presence of α-ketoglutarate and one of the amino acids Ile, Leu, Val, Phe or Met, while the same cells lacking CitP showed transamination activity only after permeabilization of the cell membrane. Moreover, the transamination activity of the cells followed the levels of CitP in a controlled expression system. The involvement of CitP in the uptake of the α-keto donor was further demonstrated by the increased consumption rate in the presence of L-lactate that drives CitP in the fast exchange mode of transport. Transamination is the only active pathway for the conversion of α-ketoglutarate in IL1403; a stoichiometric conversion to glutamate and the corresponding α-keto acid from the amino acids was observed. The transamination activity by both the cells and the cytoplasmic fraction showed a remarkably flat pH profile in the range between 5-8, especially with the branched-chain amino acids. Further metabolism of the produced α-keto acids into α-hydroxy acids and other flavor compounds required the coupling of transamination to glycolysis. A much broader role of the citrate transporter CitP in LAB than citrate uptake in the citrate fermentation pathway is discussed.

Introduction

Transamination is the central reaction in amino acids catabolic pathways in Lactic Acid Bacteria (LAB) that results in the formation of aroma compounds in food fermented products. The reaction is catalyzed by aminotransferases and results in the formation of the corresponding α-keto acids from amino acids while an α-keto donor (f.i. α-ketoglutarate) is transformed to the corresponding amino acid (i.e. glutamate). Subsequently, α-keto acids are degraded to flavor compounds like aldehydes, alcohols, carboxylic acids or α-hydroxy acids, by additional (enzymatic or chemical) reactions. α-Ketoglutarate is reported to be the best keto donor for transamination by the common dairy starter bacterium *Lactococcus lactis* (28, 29). Two major α-ketoglutarate transaminases involved in flavor development in *L. lactis* have been identified and characterized. BcaT converts branched chain amino acids and methionine (28), AraT converts aromatic amino acids, methionine, and leucine (21). A double knock-out of the two encoding genes *bcaT* and *araT*, respectively, showed that another transaminase is involved in the conversion of aspartate (23). The latter is transaminated by aspartate transaminase AspC encoded by the *aspB* gene in *L. lactis* IL1403 (2, 6). All three enzymes belong to the same family of pyridoxal 5′-phosphate (PLP) dependent transaminases.

The conversion of amino acids into aroma compounds by LAB in cheese is often limited by the lack of α-ketoglutarate. Only a few strains of *L. lactis* are capable of producing α-ketoglutarate by deamination of glutamate, which is present in large quantities in milk, catalyzed by glutamate dehydrogenase (22). The other alternative to enhance cheese aroma would be addition of external α-ketoglutarate (23). Unfortunately, external α-ketoglutarate must be provided at high concentration to result in enhanced flavor compounds production, possibly because of the lack of an uptake system for α-ketoglutarate.

CitP, the citrate uptake system in LAB was previously characterized in *L. lactis* (13, 17, 18) and *Lc. mesenteroides* (1, 14). The primary function of CitP is uptake of citrate during citrate/ carbohydrate cometabolism when CitP catalyzes the uptake of citrate into the cell in exchange with the end product of glycolysis L-lactate (precursor/product exchange) (1, 14). Studies in resting cells showed that under conditions when L-lactate was not present, citrate was taken up by CitP in exchange with the citrate metabolic pathway intermediates/end products: pyruvate, α-acetolactate, and/or acetate (17). Similar studies demonstrated the affinity of CitP for oxaloacetate, another intermediate of citrate metabolism, by identifying the role of CitP in oxaloacetate metabolism (17) and citrate driven transamination in an oxaloacetate decarboxylase deficient mutant of *L. lactis* IL1403 (18). The different metabolic functions of CitP were consistent with previous citrate transport studies *in vitro* using right-side-out (RSO) membrane vesicles derived from *L. lactis*, in which the transporter was demonstrated to translocate 2-hydroxycarboxylates (citrate, L-lactate, α-acetolactate) and 2-ketocarboxylates (pyruvate and oxaloacetate) (1), but also showed a new substrate, the carboxylate acetate (17). Subsequently, a systematic study of the substrate specificity of the transporter characterized CitP as a very

promiscuous carboxylate transporter that translocates mono-, di- and tri-carboxylates of the form $X\text{-}CR_2\text{-}COO^-$ in which X is either OH, O, or H but not NH_3^+ (amino acids), and with a preference for OH>O>H at the C2 atom (19). Many of the monocarboxylate substrates are flavor compounds or precursors thereof derived from amino acid catabolic pathways, i.e. α-keto acids and α-hydroxy acids. In addition, many metabolites like the hydroxy (OH), keto (O), and acid (H) forms of C4-dicarboxylates (i.e. L-malate, oxaloacetate, succinate) and C5- and C6-dicarboxylates (glutarate and adipate, respectively) are transported by CitP (1, 19).

In the present study, it was first demonstrated that α-ketoglutarate driven transamination by *L. lactis* IL1403 is limited by the lack of an uptake system for α-ketoglutarate. Subsequently, it is shown that the limitation is overcome in *L. lactis* IL1403(pFL3), a recombinant strain that expresses the citrate transporter CitP. CitP is shown to recognize and catalyze the uptake of α-ketoglutarate into the cell. The study emphasizes the potentially broad role of the citrate transporter CitP in flavor compound production by *Lactococcus lactis* subsp. *diacetylactis.*

Materials and methods

Chemicals

2-hydroxy-4-methylthiobutyrate, 2-keto-3-methylvalerate, 2-keto-4-methylthiobutyrate, aldehyde dehydrogenase, diethylethoxymethylenemalonate (DEEMM), L-alanine, L-aminoadipic acid, L-cysteine, L-glutamine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, L-valine, methyl-3-methylthiopropionate, nisin, phenyllactate, phenylpyruvate, pyridoxal 5′-phosphate (PLP), α-ketoglutarate, α-ketoisocaproate, and α-ketoisovalerate were obtained from Sigma Aldrich Chemicals. L-arginine, L-asparagine, L-aspartate, L-glutamate, L-glycine, and L-methionine were obtained from Merck. Solutions were prepared in 50 mM potassium phosphate pH 5.8 buffer; if required, the pH was adjusted to 5.8 with 5 M KOH.

Bacterial strain and growth condition

Strains *Lactococcus lactis* IL1403 (4), IL1403(pFL3) (13), and IL9000(pNZ-CitP) (16) were used in this study. Plasmid pFL3 harbours the lactococcal CRL264 *citP* gene under control of the *Streptococcus pneumoniae polA* promoter (13). Neither expression nor plasmid copy number is under control of citrate or pH in this strain (9). *L. lactis* IL9000 is an IL1403 derivative in which the NICE system for nisin-inducible gene expression *nisRK* is employed (16). *citP* gene in pNZ8048 plasmid is under control of the nisin-inducible promoter *nisA* (5). Precultures were grown overnight at 30 ^oC in M17 broth medium supplemented with 0.5 % (w/v) glucose (M17G) and 5 $\mu g.ml^{-1}$ of tetracycline or chloramphenicol, if required. Cells were grown in M17G medium with an initial pH adjusted to 7.0. If required, nisin was added at different concentrations ranging from 0 to 10 $ng.ml^{-1}$. Growth was performed in 100 ml serum bottles without agitation and at 30 ^oC. Growth

was followed by measuring the optical density at a wavelength of 660 nm. Cells were harvested at mid-exponential growth phase when the optical density was 0.6 by centrifugation for 10 min at 3000 rpm. Cells were washed two times with 50 mM potassium phosphate pH 5.8 buffer, centrifuged at 4 ^{o}C, and finally, resuspended in the same buffer at 4 ^{o}C.

α-Ketoglutarate driven transamination

Resting cells at an OD_{660} of 1.5 in 50 mM potassium phosphate pH 5.8 buffer were incubated at 30 ^{o}C without agitation for 10 min. The assay was performed in a total volume of 1.5 ml. At t=0, 2 mM of α-ketoglutarate was added in the presence or absence of 2 mM amino acid and 50 μM PLP. Samples of 100 μl were taken every 10 or 30 min for 3 h, then after 24 h of incubation, and immediately centrifuged for 0.5 min at maximum speed in a table top centrifuge. The supernatant was stored on ice or frozen until further analysis by HPLC/RP-HPLC.

HPLC/RP-HPLC analysis

Samples were run on a Shimadzu high-speed HPLC Prominence UFLC and later analyzed using the LC Solutions 1.24 SP1 software from Shimadzu (Kyoto, Japan). α-Ketoglutarate and products of α-ketoglutarate driven transamination (α-keto acids and α-hydroxy acids) were determined by loading an aliquot of 10 μl of the supernatant on an Aminex HPX-87H anion-exchange column with dimensions 300 x 7.8 mm (Bio-Rad Laboratories, Inc., Richmond, CA) operated at 30 ^{o}C in isocratic mode using 0.005 M H_2SO_4 as the mobile phase and a flow rate of 0.8 ml/min.

Amino acids were analyzed by RP-HPLC after DEEMM derivatization described before (20). Briefly, aminoenone derivatives of amino acids were obtained by reaction of supernatant with 1 M borate pH 9.0 buffer, methanol, and DEEMM in closed tube over 30 min incubation at room temperature in an ultrasound bath. Then, the sample was incubated at 70 ^{o}C to allow complete degradation of the excess of DEEMM. Detection of aminoenone derivatives was performed in an Alltech Platinum EPS C18 column with dimensions 250 x 4.6 mm operated at 25 ^{o}C through the binary gradient with a flow rate 0.8 ml/min. Eluent A was 25 mM acetate pH 5.8 buffer with 0.02 % sodium azide and eluent B was 80:20 mixture of acetonitrile and methanol. The target compounds were identified according to the retention times and were quantified using the external standard method. Standard deviations were obtained from at least 3 different experiments.

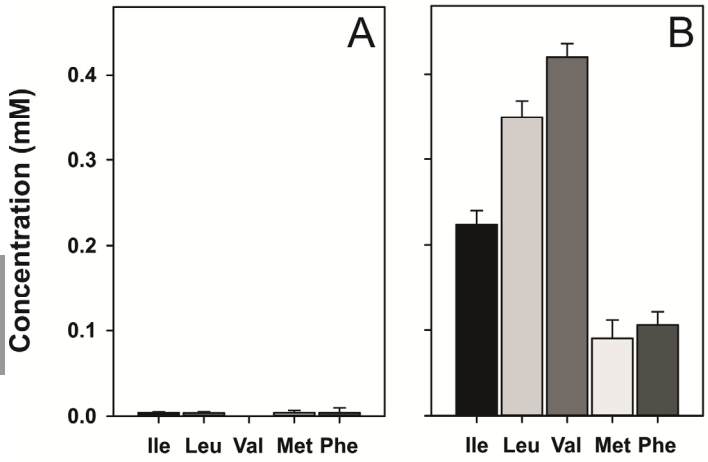
Results

Transamination of α-ketoglutarate in L. lactis IL1403

Transamination activity by *L. lactis* IL1403 with α-ketoglutarate as an external keto donor was studied by incubating resting cells with 2 mM of α-ketoglutarate in the presence of 50 μM of the

cofactor PLP and 2 mM of the amino acids Ile, Leu, Val, Phe, or Met. No α-keto acids were formed from any of these amino acids after 3 h of incubation (Fig. 1A). The experiment was repeated after permeabilization of the cells which omits all transport steps. Cells were permeabilized by treatment with the detergent Triton X-100 at a concentration of 0.15 % for 20 min just before addition of α-ketoglutarate and each of the five amino acids. The corresponding α-keto acids of Ile, Leu, Val, Met, and Phe were formed after 3 h at concentrations of 230, 350, 420, 100, and 100 μM, respectively (Fig. 1B). Almost complete conversion of α-ketoglutarate was observed after 24 h of incubation in the presence of the branched-chain amino acids, whereas conversion of Met and Phe was about two times slower (Table 1). The amounts of α-ketoglutarate consumed and α-keto acids produced were in good agreement indicating that other pathways for α-ketoglutarate conversion were not active in permeabilized cells of *L. lactis* IL1403. Control experiments performed in the absence of α-ketoglutarate did not result in the formation of the α-keto acids and omitting PLP from the incubation mixture strongly decreased the production of the α-keto acids (not shown). Hence, PLP-dependent transaminases are active in the cytoplasm of *L. lactis* IL1403 with α-ketoglutarate as the keto donor and the amino acids Ile, Leu, Val, Phe, and Met. Most importantly, α-ketoglutarate driven transamination by resting cells was limited by the uptake and/or excretion of one or more of the substrates and products. Since no transamination was observed in resting cells with all 5 amino acids and a previous study showed no limitation in import/export of amino acids (20), the limitation is most likely in the uptake of α-ketoglutarate.

Figure 1. α-Ketoglutarate driven transamination by resting (A) and permeabilized (B) cells of *L. lactis* IL1403.



Concentrations of the corresponding α-keto acids from Ile, Leu, Val, Phe, and Met were determined after 3 h of incubation of the cells in the presence of 2 mM α-ketoglutarate, 2 mM of amino acids, and 50 μM of PLP.

α-Ketoglutarate driven transamination by L. lactis IL1403(pFL3)

α-Ketoglutarate driven transamination was assayed in resting cells of *L. lactis* IL1403(pFL3) that expresses the citrate transporter gene *citP* located on plasmid pFL3 (13). Plasmid pFL3 contains

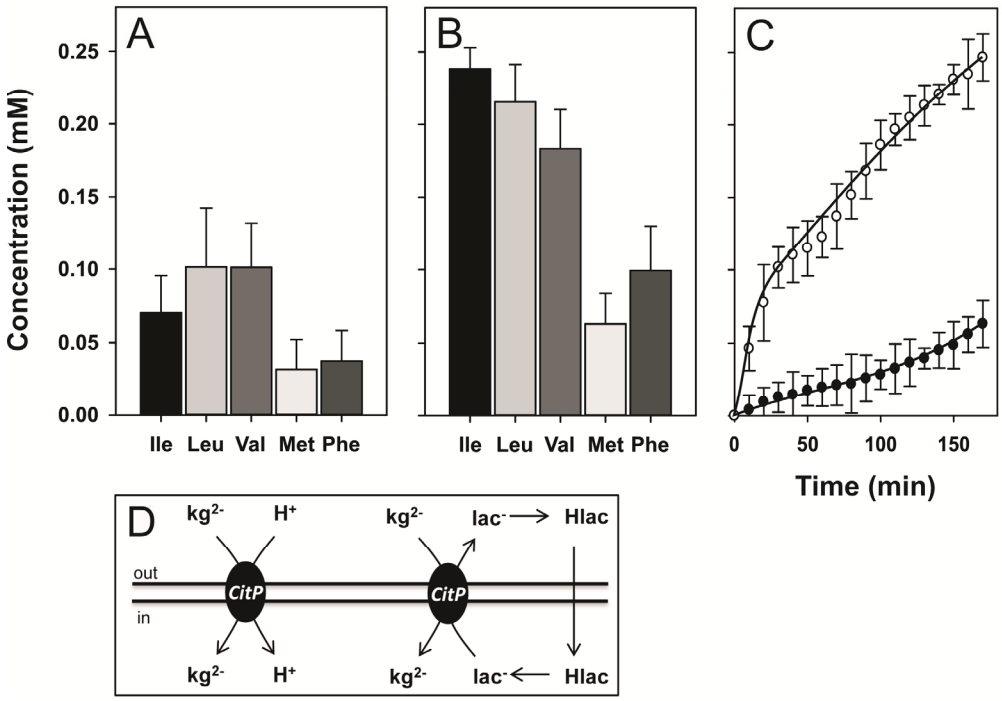
Table 1. Efficiency of α-ketoglutarate driven transamination by *L. lactis* IL1403(pFL3).

	Incubation (h)	aa[a]	α-kg (μM)[b]	α-ka (μM)[c]	Efficiency[d] (%)
L. lactis IL1403(pFL3) (resting cells)	3	Ile	96 ± 6	92 ± 7	96 ± 13
		Leu	97 ± 8	96 ± 3	99 ± 11
		Val	95 ± 5	96 ± 6	99 ± 11
		Met	39 ± 5	39 ± 5	100 ± 25
		Phe	44 ± 5	44 ± 5	100 ± 23
	24	Ile	930 ± 93	985 ± 84	106 ± 19
		Leu	1023 ± 101	1011 ± 89	99 ± 19
		Val	1159 ± 87	1117 ± 88	96 ± 15
		Met	471 ± 82	457 ± 52	97 ± 28
		Phe	411 ± 52	417 ± 62	101 ± 28
L. lactis IL1403/IL1403(pFL3)[e] (permeabilized cells)	3	Ile	254 ± 27	224 ± 19	88 ± 18
		Leu	331 ± 30	353 ± 32	106 ± 19
		Val	442 ± 52	420 ± 29	95 ± 18
		Met	111 ± 12	101 ± 11	91 ± 21
		Phe	95 ± 11	110 ± 13	115 ± 25
	24	Ile	1710 ± 68	1802 ± 70	105 ± 8
		Leu	1821 ± 107	1826 ± 101	100 ± 11
		Val	1920 ± 96	1798 ± 98	94 ± 10
		Met	1069 ± 57	1066 ± 87	100 ± 13
		Phe	1040 ± 67	1100 ± 99	105 ± 16

[a] amino acids were added at the concentration of 2 mM; [b] amount of α-ketoglutarate consumed; [c] amount of α-keto acid produced from the amino acid; [d] percentage of α-ketoglutarate converted to the α-keto acid; [e] similar activities were observed with the two strains; the data was pooled.

the lactococcal *citP* gene under control of the constitutive promoter *polA* from *S. pneumoniae* (13). Neither expression level nor plasmid copy number are affected by the presence of citrate or the pH of the growth medium (9), resulting in a constant level of expression of CitP. In contrast to strain IL1403, resting cells of IL1403(pFL3) produced significant amounts of α-keto acids when incubated with α-ketoglutarate in the presence of each of the amino acids Ile, Leu, Val, Met or Phe and PLP (Fig. 2A). After 3 h, 90-100 μM of α-ketomethylvalerate, α-ketoisocaproate, and α-ketoisovalerate was produced in the presence of Ile, Leu, and Val, respectively, while in the presence of Met and Phe the amounts were lower, 40-45 μM of 2-keto-4-methylthiobutyrate and phenylpyruvate, respectively. The amounts increased 10-fold after 24 h of incubation (Table 1). Similarly as observed with permeabilized cells of IL1403, the amounts of α-ketoglutarate consumed and α-keto acids produced at both time points were in good agreement suggesting no other pathway for α-ketoglutarate, except for transamination, in resting cells as well. No formation of α-keto acids was observed without addition of a keto donor (not shown). Transamination

Figure 2. α-Ketoglutarate driven transamination in resting cells of *L. lactis* IL1403(pFL3).



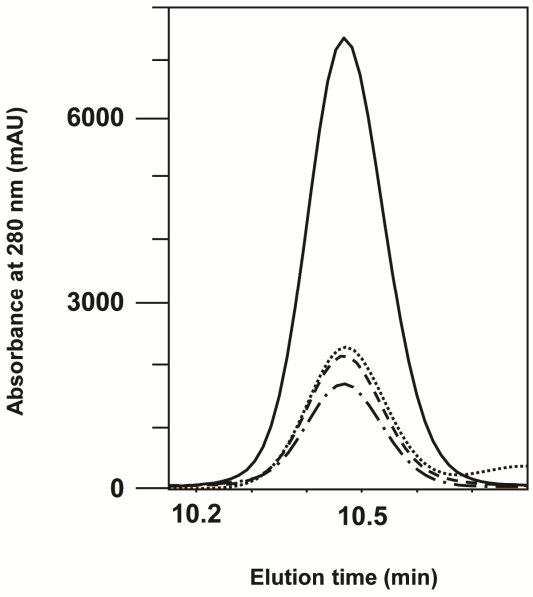
A, B. Concentrations of the corresponding α-keto acids from Ile, Leu, Val, Phe, and Met were determined in the absence (A) and presence (B) of 0.2 mM L-lactate after 3 h of incubation with 2 mM α-ketoglutarate, 2 mM of amino acids, and 50 μM of PLP. C. Production of α-ketomethylvalerate, the α-keto acid of Ile, in the absence (●) and the presence (○) of 0.2 mM L-lactate. Cells were incubated in the presence of 2 mM α-ketoglutarate, 2 mM Ile, and 50 μM of PLP. D. Kinetic modes of the citrate transporter CitP. Left, slow unidirectional uptake of divalent α-ketoglutarate (kg^{2-}) in symport with a H^+. Right, the L-lactate shuttle mechanism. L-lactate added to the outside of the cells allows CitP to operate in the fast kg^{2-}/lac^- exchange mode by reentering the cell in the permeative protonated state.

activity of permeabilized cells of the recombinant and parental strain were not significantly different (see Table 1).

Production of Glu by resting cells of the recombinant strain under identical conditions as described above was measured by RP-HPLC (see Materials and methods). Cells incubated for 3 h without further additions, with 2 mM α-ketoglutarate or with 2 mM of Ile, produced 30-40 μM of Glu (Fig. 3). In addition, low concentrations (~10 μM) of Asp, Asn, Gln, Gly, Thr, and Lys were detected (not shown) suggesting some proteolytic activity of the cells as was observed before (20). In the presence of both α-ketoglutarate and Ile, the amount of Glu produced by the cells was raised significantly to 120 μM (Fig. 3 and Table 2). The same experiment was performed with the other 4 amino acids Leu, Val, Phe, and Met, and in all cases, an increase in the production of Glu was observed when both α-ketoglutarate and the amino acid were present (Table 2). The amounts of α-ketoglutarate consumed and Glu and α-keto acids produced were in good agreement (Table 1 and 2). Hence, resting cells of *L. lactis* IL1403 expressing the citrate transporter CitP revealed enhanced transamination activity driven by external α-ketoglutarate.

The citrate transporter CitP operates in two kinetic modes, symport with a H^+ and exchange with L-lactate, which is the mode of transport during carbohydrate/citrate cometabolism. The exchange mode is much faster than the symport mode which results in a strong enhancement of the citrate consumption rate by resting cells in the presence of a low concentration of L-lactate (1, 14, 17). Then, L-lactate is involved in a futile cycle in which it enters the cell passively in the permeative, protonated form and leaves the cell again in exchange with citrate catalyzed by CitP ('shuttle mechanism') (see also Fig. 2D). Enhancement of the uptake rate of a substrate by L-lactate is diagnostic for substrates of CitP as was demonstrated before also for oxaloacetate (18). In the presence of 0.2 mM L-lactate, resting cells of *L. lactis* IL1403(pFL3) incubated with 2 mM α-ketoglutarate and 2 mM of the amino acids showed an increased production of the

Figure 3. Glutamate production during α-ketoglutarate driven transamination by resting cells of *L. lactis* IL1403(pFL3).



Resting cells were incubated for 3 h without further additions (– – – –), in the presence of 2 mM α-ketoglutarate (• — • — •), 2 mM Ile (- - - - - -), and 2 mM α-ketoglutarate and 2 mM Ile (———). 50 μM of PLP was present in all experiments. The graphs represent the RP-HPLC elution profile.

Table 2. α-Ketoglutarate driven transamination in *L. lactis* IL1403(pFL3).

aa[a]	Glu (μM)[b]
Ile	95 ± 5
Leu	95 ± 5
Val	98 ± 5
Phe	63 ± 5
Met	35 ± 8

[a] amino acids were added at the concentration of 2 mM;
[b] amount of Glu was measured after 3 h and corrected for the amounts produced by proteolytic activity.

corresponding α-keto acids by at least a factor of 2 after 3 h (Fig. 2B). Detailed kinetics of the production of α-ketomethylvalerate from Ile in the presence and absence of L-lactate showed that the rate of formation was significantly higher from the beginning in its presence (4.0 and 0.5 μM.min^{-1}, respectively) (Fig. 2C). No α-keto acids were formed when the cells were incubated with L-lactate and the amino acids in the absence of α-ketoglutarate (not shown). Also, cells of *L. lactis* IL1403 not expressing CitP did not show formation of α-ketomethylvalerate when incubated with α-ketoglutarate, L-lactate, and the amino acids.

The experiments clearly demonstrate that CitP is responsible for the uptake of α-ketoglutarate in the transamination activity catalyzed by cells of IL1403 (pFL3). Comparison of the amounts of α-keto acids formed by resting cells of IL1403(pFL3) and by the same cells permeabilized with Triton X-100 demonstrated that even in the presence of L-lactate, uptake of α-ketoglutarate limits the rate of the reaction (see Table 1 and Fig. 2).

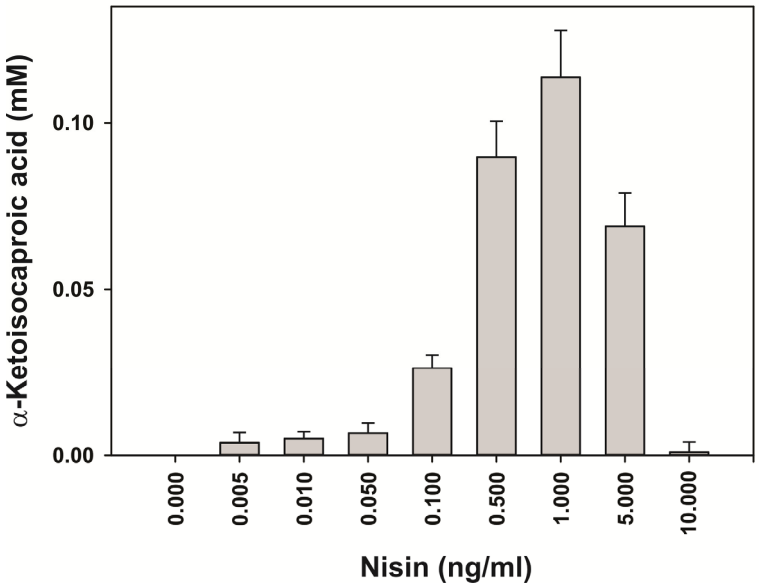
Optimizing of α-ketoglutarate driven transamination by controlled expression of CitP

Recently, the nisin inducible expression system was transferred from *L. lactis* strain MG1363 to strain IL1403 (16). Strain IL9000(pNZ-CitP), a derivative of IL1403(pFL3), contains the *citP* gene under control of the nisin-inducible promoter *nisA* (see Materials and methods). IL9000(pNZ-CitP) was grown till a density of OD_{660}=0.6 in the presence of nisin at concentrations ranging from 0 to 10 ng.ml^{-1}. Production of α-ketoisocaproic acid by resting cells incubated with 2 mM of α-ketoglutarate and Leu was measured after 3 h (Fig. 4). The transamination activity was optimal when the cells were grown in the presence of 0.5-5 ng.ml^{-1} of nisin with the highest production of the α-keto acid (115 μM) at 1 ng.ml^{-1} of nisin. Cells grown in the absence of the inducer did not produce any α-ketoisocaproic acid. Similarly, no production was observed at higher inducer concentrations. Cells growing under these conditions are severely sick and, probably, produce inactive CitP. It follows that the transamination activity of the cells follows the expression level of CitP. Unfortunately, the amount of α-ketoisocaproic acid produced by resting cells of IL1403 (pFL3) and IL9000(pNZ-CitP) growing in the presence of 1 ng.ml^{-1} nisin was similar (Fig. 2B and 4) suggesting similar levels of expression with the two expression systems.

Effect of glucose on further metabolism of α-keto acids

Enhancement of citrate (and oxaloacetate) consumption rate by L-lactate was also observed

Figure 4. Production of α-ketoisocaproate, the α-keto acid of Leu, by resting cells of *L. lactis* IL9000(pNZ-CitP).



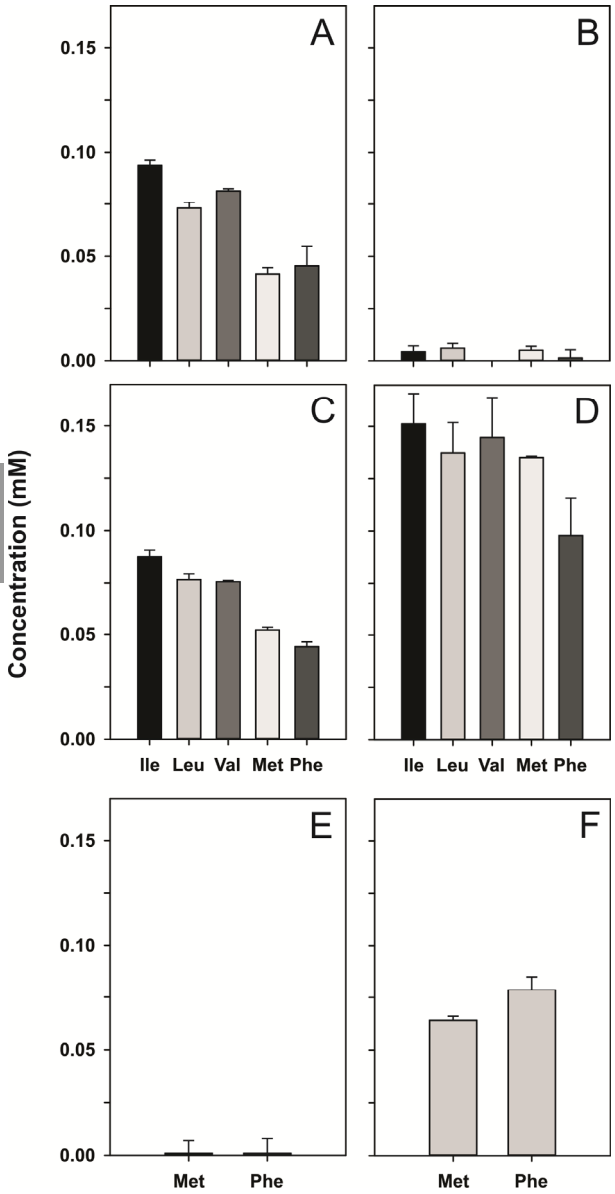
Cells were grown till OD_{660}=0.6 in the presence of nisin added to the growth medium from the beginning at concentrations between 0-10 ng.ml^{-1}. Resting cells were incubated for 3 h in the presence of 2 mM α-ketoglutarate, 2 mM Leu, and 50 µM of PLP.

when L-lactate was produced in the cytoplasm by glycolysis in the presence of glucose (17, 18). Surprisingly, resting cells of IL1403(pFL3) produced much lower amounts of α-keto acids after 3 h when 1 mM of glucose was included to α-ketoglutarate and the amino acids (Fig. 5A, B). At the same time, the amount of Glu produced by the cells was significantly higher than observed with the cells incubated in the absence of glucose (Fig. 5C, D) suggesting high transamination activity. In the absence of glucose, the amounts of the α-keto acids and Glu produced were in good agreement for all tested amino acids (Fig. 5A, C). The data suggests that in the presence of glucose, the α-keto acids were not the end products. Reduction of α-keto acids to α-hydroxy acids by resting cells of *L. lactis* was previously observed after addition of glucose (24) and during citrate driven transamination in an oxaloacetate decarboxylase mutant (20). Accordingly, α-hydroxy acids formed from Met and Phe, i.e. 2-hydroxy-4-methylthiobutyrate or phenyllactate, respectively, were measured in the presence of glucose and not in its absence (Fig. 5E, F). α-Hydroxy acids derived from branched-chain amino acids could not be measured due to limitations of the HPLC detection method but the high production of Glu strongly suggests further metabolism of the corresponding α-keto acids (Fig. 5D). The amounts of 2-hydroxy-4-methylthiobutyrate and phenyllactate were lower than the amounts of Glu produced indicating additional conversion of the α-keto acids into other flavor compounds (Fig. 5D, F). No conversion of the α-keto acids produced by permeabilized cells was observed when glucose or NADH was added to the incubation mixture (not shown). In conclusion, glucose metabolism provides the reducing equivalents and, possibly, the proper conditions in the cell to improve formation of aroma compounds.

pH dependence of α-ketoglutarate driven transamination

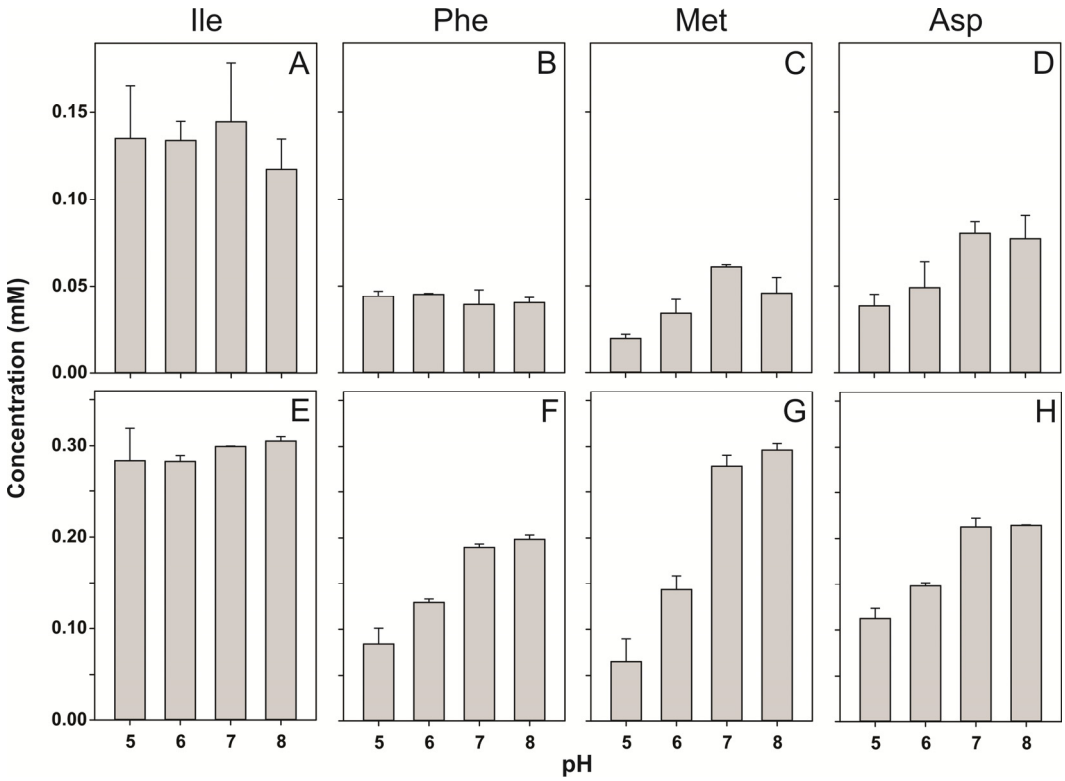
BcaT and AraT, the two major transaminases of *L. lactis* that show affinity towards branched-chain amino acids, aromatic amino acids, and Met were reported to be most active in the pH range between 7-8, and therefore, most studies were performed at an external pH of 8 (21, 28). This is surprising, since the cytoplasmic pH of *L. lactis* IL1403(pFL3) under conditions where no energy source like glucose is present is close to 6 (17, 18). The pH profile of the aspartate transaminase AspC has not been studied (6). Here, Ile was chosen as a representative for BcaT, Phe for AraT, Met for BcaT and AraT, and Asp for AspC. The α-ketoglutarate driven transamination activity by resting cells of *L. lactis* IL1403(pFL3) was determined in the pH range from 5 to 8 by measurement of the formation of the corresponding α-keto acid (Fig. 6). The pH profile was remarkably flat for the process as a whole including the transport steps (Fig. 6A-D). Only with Met and Asp a slightly lower activity was observed at the acidic side of the spectrum. Bypassing of the transport steps by treating the cells with Triton X-100 revealed the same flat profile for Ile which is representative for BcaT activity (Fig. 6E). Conversion of Phe and Asp catalyzed by AraT and AspC, respectively, is slightly raised at higher pH (Fig. 6F, H). In line with a previous studies (28), formation of the α-keto acid of Met was improved at high pH (Fig. 6G). The final amounts

Figure 5. α-Ketoglutarate driven transamination in resting cells of *L. lactis* IL1403(pFL3) in the absence (A, C, E) and presence of 1 mM glucose (B, D, F).



Concentrations of the corresponding α-keto acids (A, B), Glu in the presence of Ile, Leu, Val, Phe, and Met (C, D), and the α-hydroxy acids of Met and Phe (E, F) were determined after 3 h of incubation with 2 mM α-ketoglutarate, 2 mM of the amino acids, and 50 μM of PLP.

Figure 6. α-Ketoglutarate driven transamination in resting (A-D) and permeabilized cells (E-H) of *L. lactis* IL1403(pFL3) with Ile (A, E), Phe (B, F), Met (C, G), and Asp (D, H).



The corresponding α-keto acids were determined after 3 h of incubation in 50 mM potassium phosphate buffer at the indicated pH containing 2 mM α-ketoglutarate, 2 mM of the amino acid, and 50 µM of PLP.

formed of each α-keto acid after 24 h were equal over the whole pH range in whole and permeabilized cells (not shown). The production of α-hydroxy acids from Met and Phe at high pH in the presence of glucose was completely inhibited (not shown). Summarizing, the external pH does not affect the transamination activity of the cells *in vivo*, but further conversion into flavor compounds may be more effective in the lower, more physiological pH range.

Discussion

Transamination in L. lactis driven by external α-ketoglutarate

α-Ketoglutarate is the preferred keto donor in the reaction catalyzed by lactococcal transaminases that catalyze the first step in flavor development from excess amino acids in dairy fermentations. Often, the availability of cytoplasmic α-ketoglutarate limits the production of flavor compounds, a limitation that is only partly overcome by adding the keto donor to the external medium (26, 27, 30). The present study clearly shows that the lack of an uptake system in the cytoplasmic membrane of strain IL1403 is the reason for this failure. While resting cells did not catalyzed

transamination in the presence of α-ketoglutarate and a number of amino acids, significant activity was observed after disruption of the membrane demonstrating the necessity of an uptake system to make α-ketoglutarate accessible to the transaminases in the cytoplasm. Surprisingly, it was demonstrated that the citrate transporter CitP found in specific strains of *L. lactis* is capable of transporting α-ketoglutarate into the cell by three criteria, (i) strain IL1403 harboring plasmid pFL3 encoding CitP revealed significant transamination activity (Fig. 2), (ii) transamination activity of strain IL1403 followed the controlled expression of the *citP* gene from plasmid pNZ-CitP that contains the gene under control of the inducible nisin promotor (Fig. 4), and (iii) the rate of consumption of external α-ketoglutarate by IL1403 containing CitP was enhanced in the presence of L-lactate added directly or produced by glycolysis (Fig. 2 and 5). The latter observation is diagnostic for substrates of CitP and relates to the very efficient citrate/L-lactate exchange mode of transport catalyzed by CitP during citrate fermentation. It follows that specifically *L. lactis* strains subsp. *lactis* biovar *diacetylactis,* species that ferment citrate, should be used for flavor enhancement by externally added α-ketoglutarate. At least in resting cells, conversion of α-ketoglutarate in the transamination reactions appears to be the only pathway which assures a high efficiency of conversion into flavor compounds. The requirement for a high pH for optimal transamination activity reported before (21, 28) was not substantiated in the present study. In fact, at the relatively acidic pH values found in food fermentations, the overall activity by the cells was significant with all amino acids tested (Fig. 6). Remarkably, in the presence of only α-ketoglutarate and the amino acids, the corresponding α-keto acids were the only products formed in addition to glutamate (Table 1). Further metabolism required the presence of glucose (Fig. 5). While this is apparent for the reduction to the α-hydroxy acids for which glycolysis provides the reducing equivalents, other conversions apparently rely on specific cytoplasmic factors or conditions as well.

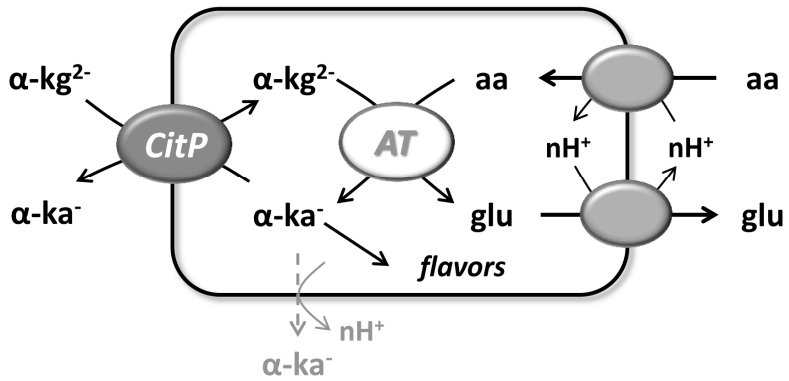
Physiological functions of the citrate transporter CitP

In citrate fermenting *L. lactis* subsp. *diacetyllactis*, the gene encoding the citrate transporter CitP is located on an endogenous plasmid, while the metabolic enzymes are clustered in a single operon on the chromosome. Moreover, expression of the transporter gene is controlled by the pH of the growth medium rather than the presence of citrate in the medium. Both arguments argue for a wider physiological function for CitP in *Lactococcus* species than the uptake of citrate in the citrate metabolic pathway alone. To contrast the situation in *Lactococcus* sp., the expression of the *citP* gene in *Leuconostoc mesenteroides* is induced by citrate and *citP* is part of the same operon that contains the metabolic enzymes (14). For sure, uptake of citrate in exchange with the metabolic end product L-lactate during citrate/carbohydrate cometabolism is also in lactococci the most prominent function of CitP. In the absence of a carbohydrate (and L-lactate), CitP catalyzes the uptake of citrate in exchange with intermediates and/or end products of the citrate metabolic pathway like pyruvate, α-acetolactate, and acetate (17). CitP functions in oxaloacetate

metabolism by taking up oxaloacetate from the medium after which it is degraded to acetate (18). Under conditions where oxaloacetate decarboxylase activity is compromised, internalized oxaloacetate was shown to function as α-keto donor in transamination reactions resulting in flavor compound production (20). The present study demonstrates the involvement of CitP in flavor producing pathways driven by α-ketoglutarate (Fig. 7). The pathway consist besides CitP of amino acid transporters and transaminases. Overall, external α-ketoglutarate and an external amino acid are converted to Glu and the α-keto acid derived from the amino acid. Alternatively, the amino acid may be produced inside by proteolytic activity. The produced α-keto acid may be excreted by CitP in exchange with α-ketoglutarate, leave the cell by passive diffusion or, be further metabolized.

The different physiological functions require that CitP has broad substrate specificity. Recently, CitP was characterized as a promiscuous carboxylate transporter (19). CitP translocates many mono-, di- and tri-carboxylates of the form X-CR_2-COO^- in which X is either OH, O, or H (19). The set of substrates includes the α-keto acids and α-hydroxy acids that are formed by transamination of amino acids. It was shown that CitP catalyzes efficient transport of C4-6 dicarboxylates such as succinate, glutarate, and adipate, and 2-hydroxy (L-malate) and 2-keto (oxaloacetate) forms of C4 -dicarboxylates (19). The present study adds α-ketoglutarate, the α-keto form of the C5-dicarboxylate glutarate to the set of substrates of CitP.

Figure 7. Schematic representation of α-ketoglutarate driven transamination in *L. lactis* IL1403 (pFL3).



α-kg, α-ketoglutarate; α-ka, α-keto acid; aa, amino acid; AT, aminotransferase; CitP, citrate permease; glu, glutamate.

Lactococcal transaminases

Metabolism of α-ketoglutarate and amino acids in *L. lactis* IL1403(pFL3) is coupled at the level of aminotransferases (Fig. 7). Two major lactococcal transaminases AraT and BcaT were purified and characterized. AraT was purified from both *L. lactis* subsp. *cremoris* NCDO763 (28) and *L.*

lactis subsp. *lactis* S3 (8). The *araT* gene from *L. lactic* subsp. *lactis* IL1403 (2) used in this study is 97% identical in sequence to the characterized enzymes. AraT is a transaminase active on aromatic amino acids (phenylalanine, tryptophan, tyrosine), leucine, and methionine utilizing α-ketoglutarate as the keto donor, activities that were also observed in the present study. Both purified enzymes were most active at pH 7-8 at optimal temperatures of 35-45 ^{o}C and 55-65 ^{o}C for enzyme from NCDO763 and S3, respectively. The cumulative transamination activities for these substrates in the cytoplasm of strain IL1403 was still high at the lower pH of 5.8 and the lower temperature of 30 ^{o}C, conditions that are more compatible to cheese production (23). BcaT purified from *L. lactis* subsp. *cremoris* NCDO763 (29) shares 99 % of sequence identity with the *bcaT* gene from *L. lactic* subsp. *lactis* IL1403. Purified BcaT is active with the branched-chain amino acids and methionine utilizing α-ketoglutarate as a keto donor (29), activities also observed in the cytoplasm of IL1403. The highest activity of the purified enzyme was observed at pH 7.5 at the optimal temperature in the range of 35-40 ^{o}C. α-Ketoglutarate driven transamination performed in IL1403(pFL3) showed that especially transamination of the branched-chain amino acid isoleucine was not sensitive to pH in the range between 5 and 8. A third transaminase annotated in the genome of strain IL1403 (2, 6), the aspartate transaminase AspC was purified from *Brevibacterium linens* 47 and shown to be active on aspartate and α-ketoglutarate with an optimal pH of 8.5-9 (12). Again, the same activity in the cytoplasm of *L. lactis* IL1403 showed a rather flat pH profile (see Fig. 6D, H). The amino acid substrate profile of α-ketoglutarate transamination activity in the cytoplasm of IL1403 reported here is very similar to the recently reported oxaloacetate transamination activity in the same strain (20), while purified AraT and BcaT did not show activity with the latter keto donor. The discrepancies between the observed activities in the cytoplasm of strain IL1403 and the properties of the purified transaminases suggest that purification alters the properties of the enzymes or that other unknown transaminases are active in IL1403.

Flavor compounds from α-ketoglutarate driven transamination

α-Keto acids derived from aromatic amino acids, branched-chain amino acids, and methionine by transamination can be further degraded to various aroma compounds either by enzymatic or chemical reactions. They may be converted to α-hydroxy acids by 2-hydroxyacid dehydrogenases (HA-DHs), to aldehydes by activity of different decarboxylases, to carboxylic acids produced by oxidative decarboxylation, and to alcohols or (thio)-esters that are produced by further metabolism of carboxylates. Most reactions and enzymes involved have been elucidated only partially (26, 30). The reduction of α-keto acids into α-hydroxy acids by resting cells of *L. lactis* was observed at the expense of NADH provided by glycolysis (Fig. 5) (24) or by citrate fermentation (20). HA-DHs are stereospecific enzymes with a broad substrate specificity that are widely distributed in LAB (7). Genome analysis of *L. lactis* IL1403 revealed the putative HA-DH gene *hicD* annotated as L-2 -hydroxyisocaproate dehydrogenase (2), the *panE* gene that was described as 2-

hydroxyisocaproate dehydrogenase with the highest catalytic efficiencies for branched-chain α-keto acids (3), and the *ldh* gene that was shown in *Lactobacillus plantarum* and *L. lactis* to be responsible for the reduction of α-keto acids derived from phenylalanine and methionine (Fig. 4F) (3, 10). α-Hydroxy acids are not considered as major flavor compounds, although they play a role in flavor development in semi-hard cheeses made with lactococci (26). The amounts of glutamate produced from α-ketoglutarate suggest that, in addition to α-hydroxy acids of methionine and phenylalanine, other aroma compounds were produced from the α-keto acids in the presence of glucose (Fig. 5D, F). Phenylpyruvate was reported to be converted to the volatile benzaldehyde (15), decarboxylated to phenylacetaldehyde (7), or converted into phenylacetate through oxidative decarboxylation (28). Benzaldehyde and phenylacetate give a bitter almond oil and sweet cherry odor, and a flowery-like odor, respectively, to fermentation products (25, 26, 30). The α-keto acid derived from methionine, 2-keto-4-methylthiobutyrate, is further converted by demethiolation into methanethiol (MTL) that is a precursor for dimethyl sulfide (DMS), dimethyl disulfide (DMDS), dimethyl trisulfide (DMTS), and dimethyl tetrasulfide (DMQS), oxidized to methylthio-acetaldehyde, or decarboxylated to methylthio-propionaldehyde (methional), a precursor for methylthio-propanol (methionol) and 3-methylthiopropionic acid (MTPA) (11). MTL, DMDS, and DMTS are strong flavor compounds with odors like rotting cabbage, onion, sulfur, and garlic (25, 26, 30). Most of these compounds are volatiles and, therefore, where not detected by the methods applied in this study.

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Chapter 7

Discussion

The aim of the study presented in this thesis was to reroute citrate metabolism into the transamination route to boost amino acids catabolism in Lactic Acid Bacteria (LAB). The studies were performed in the citrate fermenting cheese bacterium *L. lactis* subsp. *lactis* biovar *diacetylactis* IL1403(pFL3), in which the gene encoding the citrate transporter CitP is located on an endogenous plasmid (9), while the citrate metabolic enzymes are clustered on the chromosome (3). The first step in catabolism of amino acids towards flavor compounds formation that are important in the dairy industry involves transamination which requires a pool of α-keto acid to produce a range of α-keto acids from the different amino acids. Transamination is often limited by the availability of a keto donor (22, 23, 26), while amino acids are in access in the protein rich milk matrix (2). Citrate that is present in relatively high amounts in milk (17) is split by citrate lyase into acetate and oxaloacetate, further oxaloacetate is converted by oxaloacetate decarboxylase yielding pyruvate (6, 13). Both intermediates, oxaloacetate and pyruvate, are potential keto donors in the transamination reaction, but effective transamination using these α-keto acids has never been demonstrated in *L. lactis* (24, 25). Oxaloacetate was shown to accumulate to high concentrations in the cytoplasm in the oxaloacetate decarboxylase deletion strain of *L. lactis* ILCitM(pFL3) (1, 14), while it was not produced in the parental strain IL1403 (pFL3) due to high activity of oxaloacetate decarboxylase (13). Pyruvate was shown to accumulate in the cytoplasm of the parental strain (13) and to lower extend in the mutant strain due to the activity of a cryptic decarboxylase (14).

Citrate driven transamination in the mutant strain produces the α-keto acids α-ketomethylvalerate, α-ketoisocaproate, α-ketoisovalerate, phenylpyruvate, indole-3-pyruvate, *p*-hydroxyphenylpyruvate, and 2-keto-4-methylthiobutyrate corresponding to the branched-chain amino acids isoleucine, leucine, valine, the aromatic amino acid phenylalanine, tryptophan, tyrosine, and the sulfur containing amino acid methionine, respectively (Table 1). Clearly, the keto donor in this transamination reaction with amino acids that are precursors of the most valuable flavors for dairy industry was shown to be oxaloacetate, and not pyruvate (15). The produced α-keto acids are the precursors of many aroma compounds, like aldehydes, alcohols, carboxylic acids, (thio)-esters, or α-hydroxy acids that are formed by subsequent enzymatic or chemical reactions, like decarboxylation, dehydrogenation, or oxidation (21, 22, 26). α-Keto acids produced by oxaloacetate transamination *in vivo* were further converted to aroma compounds such as α-hydroxy acids 2-hydroxy-4-methylthiobutyrate and phenyllactate from methionine and phenylalanine, respectively, and aldehyde of methionine 3-methylthiopropionic acid (MTPA). α-Hydroxy acids were produced at the expense of NADH provided by citrate fermentation pathway (15). During cheese paste experiments, oxaloacetate:phenylalanine or oxaloacetate:methionine transamination in the mutant strain resulted in flavors benzaldehyde or DMDS, respectively (unpublished data). α-Keto acids and α-hydroxy acids are not major flavor compounds themselves, but they play a role in flavor development in semi hard cheeses made with lactococci. MTPA, DMDS, and benzaldehyde are major aroma compounds and give an odor of “baked

potato", "onion, sulfurous" and "bitter almond oil, characteristic sweet cherry", respectively (21, 22). In addition, α-hydroxy acids exhibit antifungal and antilisterial activities important during cheese production (4, 8). The fraction of the citrate metabolic flux going into the transamination route *in vivo* in the oxaloacetate decarboxylase deficient mutant strain is modest, at most 10 % in the case of methionine (15). However, in terms of flavor compounds production this is highly significant. A yield of 200 μM of flavor compounds within 6 h from methionine (15), one of the most important precursors of flavors in cheese manufacturing, is much higher than observed in similar studies using α-ketoglutarate as the keto donor (Table 2) (16).

During citrate driven transamination in the parental and mutant strains, pyruvate turned out to be a keto donor for some amino acids. In general, pyruvate is involved in transamination to a much lower extent than oxaloacetate, but significant production of flavor α-keto acids like *p*-hydroxyphenylpyruvate, 2-keto-4-methylthiobutyrate, and β-mercaptopyruvate from the aromatic amino acid tyrosine and the sulfur containing amino acids methionine and cysteine, respectively, was observed (Table 1). Besides aroma compounds production, pyruvate was used as a keto donor in transamination of glycine, serine, and threonine demonstrating a role of pyruvate transamination in the metabolism of these amino acids (Table 1).

Transamination of amino acids is catalyzed by pyridoxal-5'-phosphate (PLP) dependent transaminases (12). The genome sequence of *L. lactis* IL1403 contains 13 genes encoding putative transaminases (3), among which 6 enzymes are probably involved in transamination reactions and development of flavor compounds described in this thesis (see Chapter 1). Four enzymes of *L. lactis*, AraT (24), BcaT (25), AspC (5), and YtjE (11) were purified and characterized, AlaT is highly similar in sequence to the purified enzymes studied in *Corynebactrium glutamicum* (10), and SPT is purely annotated as such based on homology (20). AraT (encoded by the *araT* gene) is specific for aromatic amino acids, BcaT (encoded by *bcaT*) is specific for branched-chain amino acids, AspC (encoded by *aspB*) is an oxaloacetate transaminase, AlaT (encoded by *aspC*) is annotated as a pyruvate transaminase, and SPT (encoded by *yeiG*) is involved in glycine, serine, and threonine metabolism. YtjE (encoded by *ytjE* gene) annotated as a transaminase showed C-S lyase activity with α, γ-elimination and is involved in final flavor development from methionine and cysteine rather than its transamination (11). AraT and BcaT were previously reported to be major transaminases in cheese flavor development from aromatic amino acids, branched-chain amino acids, and methionine (18, 19, 25). The purified enzymes showed activity with α-ketoglutarate as the keto donor and neither AraT nor BcaT showed activity with oxaloacetate or pyruvate (18, 24, 25). To clarify which transaminases are involved in the transamination of the 20 natural amino acids, citrate (oxaloacetate or pyruvate) and α-ketoglutarate driven transamination was determined in the mutant ILCitM(pFL3) and the parental strain IL1403(pFL3) of *L. lactis* (Table 1). The substrate specificity of the oxaloacetate:amino acid transaminases overlap with the specificity of the α-ketoglutarate:amino acid transaminases BcaT and AraT (15, 16). It is not very likely that the oxaloacetate specific

Table 1. Transamination of 20 natural amino acids driven by citrate or α-ketoglutarate in *L. lactis* IL1403.

Strain	ILCitM(pFL3)		IL1403(pFL3)		Transaminase
Donor	Citrate		Citrate	α-Ketoglutarate	
Acceptor	Asp	Ala	Ala	Glu	
Ile	50 ± 6	6 ± 4	7 ± 2	95 ± 3	
Leu	51 ± 9	7 ± 3	6 ± 2	95 ± 3	**BcaT**
Val	56 ± 13	8 ± 7	9 ± 2	98 ± 5	
Phe	72 ± 20	5 ± 3	8 ± 3	63 ± 4	
Tyr	14 ± 6	40 ± 10	24 ± 4	98 ± 2	**AraT**
Trp	16 ± 2	6 ± 6	3 ± 2	37 ± 8	
Asp	-	6 ± 5	10 ± 5	87 ± 10	**AspC**
Glu	110 ± 23	24 ± 6	14 ± 2	-	**AlaT**
Met	104 ± 13	13 ± 10	16 ± 2	35 ± 10	**AraT**
Cys	7 ± 6	13 ± 6	22 ± 5	10 ± 5	**YtjE (?)**
Gly	2 ± 5	25 ± 6	16 ± 2	1 ± 4	
Ser	3 ± 2	19 ± 4	10 ± 1	3 ± 3	**SPT**
Thr	8 ± 3	19 ± 4	14 ± 2	1 ± 4	
Asn	4 ± 3	4 ± 3	6 ± 2	3 ± 7	
Gln	9 ± 7	5 ± 6	2 ± 2	20 ± 25	
Arg	8 ± 5	12 ± 10	2 ± 2	9 ± 9	
Pro	4 ± 3	3 ± 3	3 ± 2	5 ± 8	
Ala	5 ± 8	-	-	14 ± 2	
His	4 ± 3	7 ± 4	6 ± 3	8 ± 6	
Lys	4 ± 2	6 ± 4	7 ± 3	6 ± 4	

Transamination was performed in the presence of 50 μM of PLP, 2 mM of amino acid (acceptor), and 2 mM of citrate or α-ketoglutarate (donor) in resting cells of *L. lactis*. After 3 h of incubation, production of aspartate, alanine, or glutamate were measured with RP-HPLC as described before (14, 15) that indicated a keto donor of transamination: oxaloacetate and pyruvate (produced from citrate), or α-ketoglutarate, respectively. Amounts of Asp, Ala, and Glu were corrected for the amounts produced by proteolytic activity.

transaminases are a separate set of enzymes; therefore, BcaT and AraT in the cytoplasm of *L. lactis* are likely to have affinity for both keto donors and are major transaminases for flavor compounds development in dairy products. Probably, AraT is also involved in pyruvate:tyrosine and pyruvate:methionine transamination. AspC and AlaT are involved in oxaloacetate:glutamate (aspartate:α-ketoglutarate) and pyruvate:glutamate (alanine:α-ketoglutarate) transamination, respectively, SPT is serine:pyruvate transaminase involved in metabolism of serine, glycine, and threonine, and possibly, YtjE is involved in pyruvate:cysteine transamination.

Table 2. Flavor compounds produced from amino acids with α-ketoglutarate as an amino acid acceptor.

Strain	***L. lactis* subsp. *cremoris* NCDO763 (7)**	***L. lactis* subsp. *cremoris* TIL46 (18)**	***L. lactis* subsp. *lactis* biovar *diacetylactis* IL1403(pFL3) (16)**	***L. lactis* subsp. *lactis* biovar *diacetylactis* ILCitM(pFL3) (15)**
Donor / *Acceptor*	α-Ketoglutarate			Oxaloacetate
Ile	-	15	95	50
Leu	11	16	95	51
Val	-	11	98	56
Phe	12	15	63	72
Tyr	-	19	98	14
Trp	-	20	37	16
Met	8	17	35	104

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Chapter 8

Summary

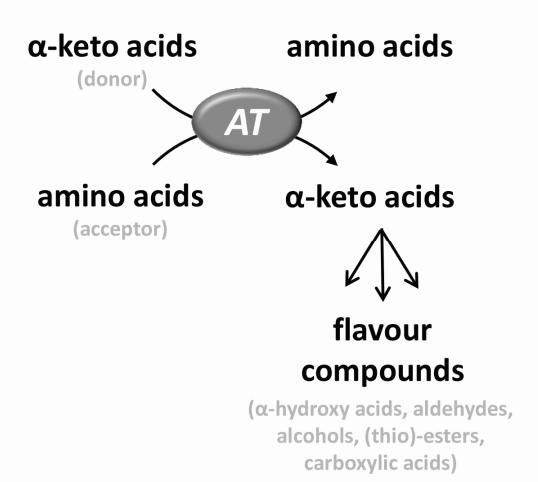
Samenvatting

Streszczenie

Summary

This thesis describes the research that I have done during the 4 years of my PhD study. The study focuses on the production of flavor compounds that are important in the dairy industry. The flavor compounds are produced from amino acids derived from milk proteins and the goal of my study was to drive the conversion by citrate in Lactic Acid Bacteria (LAB). Transamination, the first step in the breakdown of amino acids leading to production of aroma compounds (Fig. 1), is often limited by lack of an α-keto donor. A key point of the study was redirection of the first intermediate of the citrate metabolic pathway, the keto donor oxaloacetate, to the transamination reaction rather than to further be metabolized in the citrate pathway (Fig. 2). The internal pool of oxaloacetate is controlled by the enzyme oxaloacetate decarboxylase and inactivating this enzyme is proposed to do the job. Citrate is fermented by a subspecies of the cheese bacterium *Lactococcus lactis*, i.e. subsp. *lactis* biovar *diacetylactis*, in which the gene encoding the citrate transporter CitP is located on an endogenous plasmid, while the citrate metabolic enzymes are clustered on the chromosome. In this study, a model strain of *L. lactis* subsp. *lactis* biovar *diacetylactis* IL1403 with a pFL3 plasmid containing CitP was used.

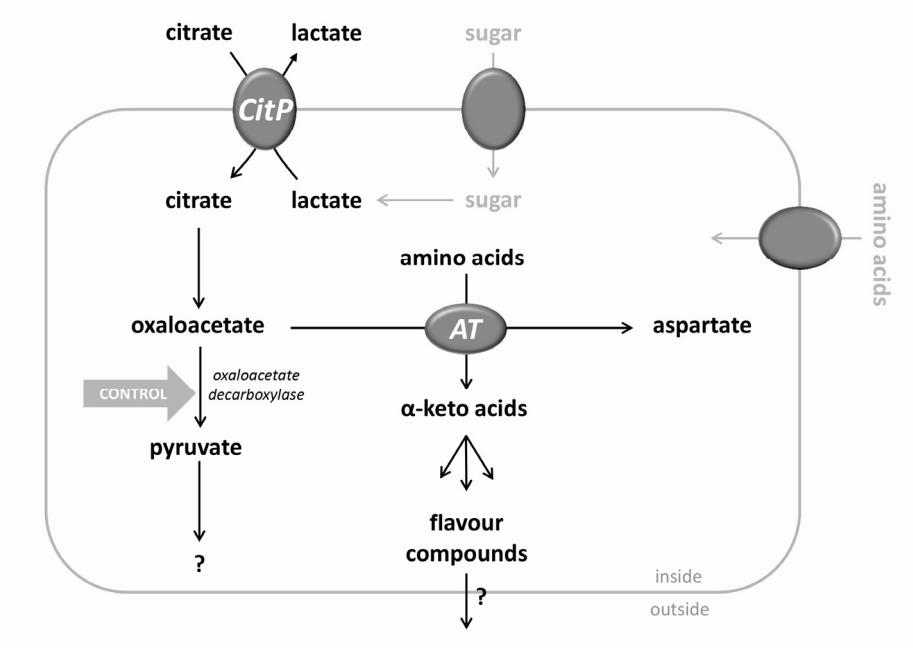
Figure 1. Schematic representation of flavor compounds production following transamination reaction.



AT, Aminotransferase (transaminase).

The first part of my study describes the characteristics of the citrate metabolic pathway in the wild -type strain of *L. lactis* IL1403(pFL3) containing CitP. The pathway splits up in two routes after pyruvate, one well-characterized to acetoin and a new pathway to acetate. Oxaloacetate, the first intermediate of citrate metabolic pathway and a potential α-keto donor for citrate driven transamination, was not observed due to the high activity of oxaloacetate decarboxylase in this strain (Chapter 2). Accumulation of oxaloacetate produced from citrate was observed in the oxaloacetate decarboxylase deletion strain derived from the wild-type. The mutant strain of *L. lactis* ILCitM(pFL3) produced high amount of internal oxaloacetate from citrate that was slowly metabolized to pyruvate by activity of a cryptic decarboxylase and further to acetate (Chapter 3). Importantly in view of the overall goal of the study, under conditions where oxaloacetate decarboxylase activity was compromised, the accumulating cytoplasmic oxaloacetate was shown to function as an α-keto donor in transamination reactions. The redirection of the citrate metabolic

Figure 2. Scheme representing production of flavor compounds by citrate driven transamination in cell of *L. lactis* subsp. *lactis* biovar *diacetylactis* IL1403 containing CitP.



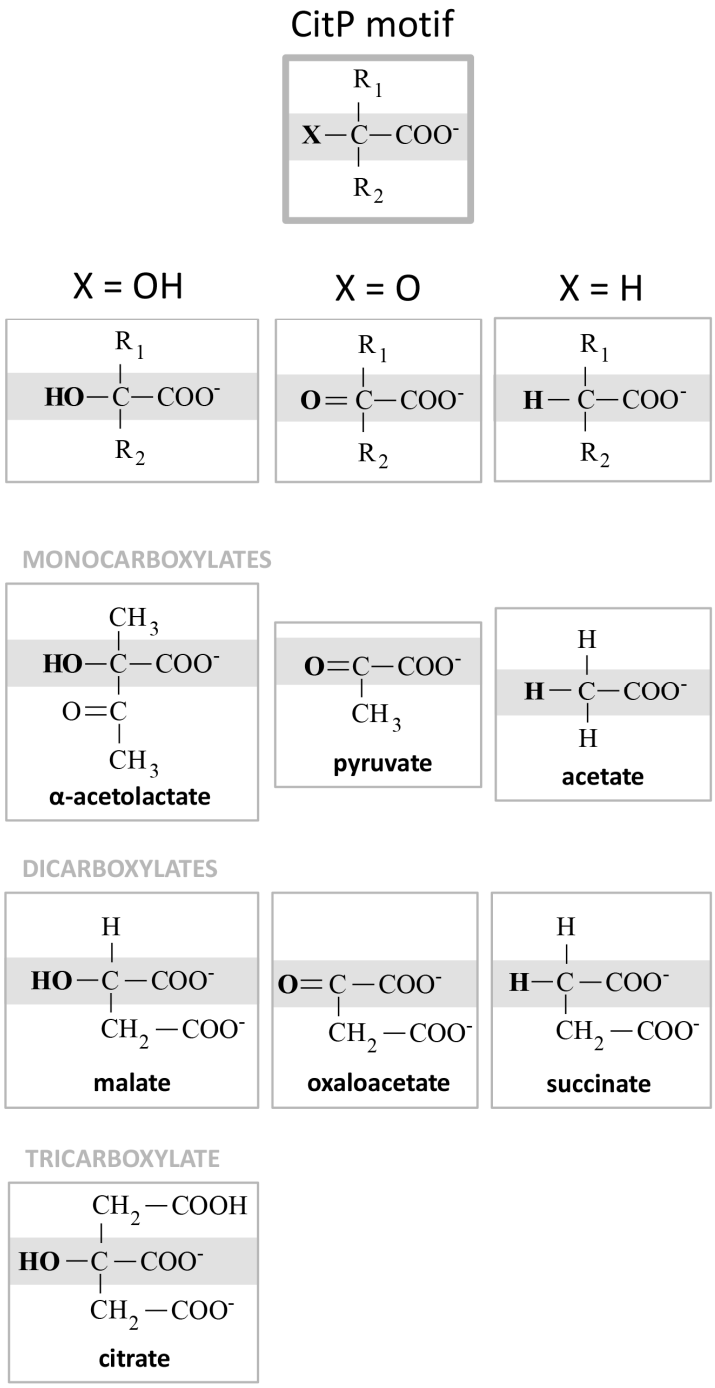
Oxaloacetate decarboxylase provides a control point to redirect citrate metabolism towards transamination (arrow). AT, aminotransferase; CitP, citrate uptake system.

pathway into the transamination route in *L. lactis* turned out to be successful resulting in flavor compounds derived from branched-chain amino acids (isoleucine, leucine, valine), aromatic amino acids (phenylalanine, tryptophan, tyrosine), and methionine (Chapter 5). A significant fraction of α-keto acids produced from methionine and/or phenylalanine were further metabolized, i.e. to α-hydroxy acid and/or to aldehyde that are considered as important flavors. This study showed that deletion of oxaloacetate decarboxylase improves citrate driven flavor formation from amino acids and demonstrates that lactococcal transaminases accept oxaloacetate as a keto donor. Another keto donor, α-ketoglutarate, which is reported as the best keto donor for transamination by *L. lactis* was studied in the wild-type strain with and without CitP. The strain IL1403(pFL3) containing CitP showed production of the corresponding α-keto acids from branched-chain amino acids, aromatic amino acids, and methionine driven by α-ketoglutarate transamination. Further metabolism of the produced α-keto acids into α-hydroxy acids and other flavor compounds that require the coupling of α-ketoglutarate transamination to glycolysis was demonstrated (Chapter 6).

The citrate uptake system CitP played a crucial role in all pathways presented in this study. For sure, the most prominent function of CitP in LAB is uptake of citrate in exchange with the metabolic end product L-lactate (precursor/product exchange) during cometabolism of citrate and a carbohydrate (see Fig. 2). In the absence of carbohydrate (and L-lactate), CitP catalyzes the

uptake of citrate in exchange with intermediates and/or end products of the citrate metabolic pathway like pyruvate, α-acetolactate, and acetate (Chapter 2). In an oxaloacetate decarboxylase deletion strain CitP has another function in citrate metabolism. Then, CitP exchanges citrate with oxaloacetate and, therefore, exports the toxic oxaloacetate from the cell. Subsequently, CitP functions in oxaloacetate metabolism by taking up oxaloacetate from the medium (Chapter 3). Finally, CitP function in the metabolism of α-ketoglutarate by taking up this compound when is present in the medium (Chapter 6). The different physiological functions require that CitP has broad substrate specificity. CitP was characterized as a very promiscuous carboxylate transporter that translocates many mono-, di-, and tri-carboxylates of the form X-CR_2-COO^- in which X is either OH, O, or H (Fig. 3) (Chapter 4). Most of CitP substrates are compounds important for the food industry. α-Keto acids and α-hydroxy acids formed by transamination of amino acids improve the quality of food fermented products. Production of these compounds by citrate (oxaloacetate) and α-ketoglutarate driven transamination is demonstrated in Chapter 5 and 6, respectively. Moreover, many of the dicarboxylates are used as food additives to enhance flavors or regulate acidity. Maybe the most important conclusion from this study is that the role of the citrate transporter CitP in LAB is much broader than just citrate uptake in the citrate fermentation pathway.

Figure 3. The motif recognized by CitP in the form of X-CR_2-COO^- in which X is either OH, O, or H.



Samenvatting

Dit proefschrift beschrijft het onderzoek dat ik uitgevoerd heb gedurende de vier jaar van mijn promotieonderzoek. Het werk richt zich op de productie van smaakcomponenten door melkzuurbacteriën die van belang zijn in de zuivelindustrie. Deze smaakcomponenten worden gevormd uit aminozuren die afkomstig zijn uit melkeiwitten, en het doel van mijn onderzoek was om die omzetting te stimuleren met citraat. Transaminatie, de eerste stap in de omzetting van aminozuren in aromatische verbindingen, wordt vaak beperkt door het ontbreken van een α-keto-donor. Een belangrijk punt van het onderzoek was het omleiden van het eerste intermediair van de route voor citraatmetabolisme, de keto-donor oxaloacetaat, naar een transaminatie-reactie in plaats van de volgende stap in de citraatroute. De interne concentratie van oxaloacetaat wordt bepaald door de activiteit van het enzym oxaloacetaatdecarboxylase en in dit proefschrift wordt gesteld dat de inactivatie hiervan de omleiding van oxaloacetaat naar transaminatiereacties kan bewerkstelligen. Citraat wordt gefermenteerd door een ondersoort van de kaasbacterie *Lactococcus lactis*, *L. lactis* subsp. *lactis* biovar *diacetylactis*, waarin het gen dat codeert voor de citraattransporter CitP gelegen is op een endogeen plasmide, terwijl de genen die coderen voor de andere metabolische enzymen in een cluster op het chromosoom liggen. In dit onderzoek is de stam *L. lactis* subsp. *lactis* biovar *diacetlylactis* IL1403 met daarin het plasmide pFL3, dat het *citP* gen bevat, gebruikt als modelstam.

Het eerste deel van mijn proefschrift beschrijft de eigenschappen van de citraatroute in de wildtype stam *L. lactis* IL1403(pFL3). De route splitst zich op na pyruvaat in een goed gekarakteriseerde route die leidt tot acetoine en een nieuwe route die leidt tot acetaat. Oxaloacetaat, het eerste intermediair in de citraatroute en een potentiële α-keto-donor voor transaminatie, werd niet aangetroffen door de hoge activiteit van oxaloacetaatdecarboxylase in deze stam (Hoofdstuk 2). In dezelfde stam waarin het gen voor de oxaloacetaatdecarboxylase uitgeschakeld was, werd echter ophoping van oxaloacetaat, als product van citraat, waargenomen. Deze mutante stam, *L. lactis* ILCitM(pFL3), produceerde een grote interne hoeveelheid oxaloacetaat dat langzaam gemetaboliseerd werd tot pyruvaat door de activiteit van een cryptische decarboxylase en daarna verder tot acetaat (Hoodfstuk 3).

Van groot belang met het oog op het uiteindelijke doel van het onderzoek was dat aangetoond is dat wanneer oxaloacetaatdecarboxylase activiteit verstoord was, het intracellulair ophopende citraat als α-keto-donor in transaminatie reacties ging functioneren. Deze omleiding van de citraatroute naar transaminatieroutes in *L. lactis* bleek succesvol, resulterend in smaakcomponenten afgeleid van isoleucine, leucine en valine (ofwel “branched-chain amino acids”), de aromatische aminozuren fenylalanine, tryptofaan en tyrosine, en methionine (Hoofdstuk 5). Een significant deel van de α-ketozuren gevormd uit methionine en/of fenylalanine werd verder gemetaboliseerd tot een α-hydroxyzuur en/of een aldehyde, die gezien worden als belangrijke smaakcomponenten. Dit onderzoek laat zien dat het verwijderen van het

oxaloacetaatdecarboxylase de vorming van smaakcomponenten, in aanwezigheid van citraat, verhoogt en dat transaminases in *L. lactis* oxaloacetaat als keto-donor accepteren. Ook α-ketoglutaraat, een ander ketozuur dat eerder beschreven werd als de beste α-keto-donor voor transaminatiereacties in *L. lactis*, werd onderzocht in de wild type stam in aan- en afwezigheid van de citraattransporter CitP. De stam IL1403(pFL3), met CitP, produceerde α-ketozuren van "branched chain" aminozuren, aromatische aminozuren en methionine door middel van transaminatie met α-ketoglutaraat als α-keto donor. Verdere metabolisering van de geproduceerde ketozuren naar α-hydroxyzuren en andere smaakcomponenten werd aangetoond en vereisen de koppeling van α-ketoglutaraat transaminatie aan glycolyse (Hoofdstuk 6).

Het opnamesysteem voor citraat, CitP, speelt een cruciale rol in alle routes die in dit onderzoek beschreven worden. De belangrijkste functie van CitP in melkzuurbacteriën is de opname van citraat in uitwisseling met lactaat ("precursor/product exchange"), het belangrijkste metabolische eindproduct van melkzuurbacteriën, tijdens cometabolisme van citraat en een koolhydraat. In afwezigheid van een koolhydraat (en L-lactaat) katalyseert CitP de opname van citraat in uitwisseling met intermediairen en/of eindproducten van de citraatroute, zoals pyruvaat, α-acetolactaat en acetaat (Hoofdstuk 2). In een oxaloacetaatdecarboxylase mutante stam heeft CitP een andere functie in citraat metabolisme. Dan wisselt CitP citraat uit tegen oxaloacetaat, waarmee het ophopende en daarmee toxische oxaloacetaat de cel uit getransporteerd wordt. Vervolgens functioneert CitP in oxaloacetaat metabolisme door citraat weer uit het medium op te nemen (Hoofdstuk 3). Ook functioneert CitP in het metabolisme van α-ketoglutaraat, door dit uit het medium op te nemen als het daar aanwezig is (Hoofdstuk 6). Deze verschillende fysiologische functies vereisen een brede substraat specificiteit van CitP. Er werd aangetoond dat CitP een vrij onspecifieke carboxylaat transporter is die vele mono-, di- en tricarboxylaten van de vorm X-CR_2-COO-, waarbij X een OH, O of H is, transporteert (Hoofdstuk 4). De meeste van de CitP substraten zijn belangrijk in de voedingsindustrie. α-Ketozuren en α-hydroxyzuren die gevormd worden door transaminatie van aminozuren verbeteren de kwaliteit van gefermenteerde voedingsproducten. De vorming van deze verbindingen door citraat (oxaloacetaat) en α-ketoglutarate gedreven transaminatie wordt aangetoond in respectievelijk Hoofdstuk 5 en 6. Bovendien worden veel van de dicarboxylaten gebruikt als voedseladditieven, als smaakversterkers of zuurteregelaars. Misschien is wel de belangrijkste conclusie van dit onderzoek dat de rol van de citraattransporter CitP in melkzuurbacteriën veel breder is dan alleen het opnemen van citraat als onderdeel van de citraat fermentatieroute.

Streszczenie

Książka ta zawiera wyniki badań, które prowadziłam w ciągu ostatnich czterech lat. Organizmem modelowym we wszystkich przedstawionych tu eksperymentach jest niepatogenna Gram-pozytywna bakteria *Lactococcus lactis*, która należy do grupy bakterii fermentacji mlekowej. Jednym z naturalnych środowisk jej występowania jest mleko, w skład którego wchodzą białka (kazeina, albuminy, immunoglobuliny), cukry (laktoza, która zbudowana jest z glukozy i galaktozy), kwas cytrynowy, substancje mineralne i witaminy. Wyselekcjonowane szczepy *L. lactis* wykorzystywane są obecnie w przemyśle mlecznym, a ich główną zaletą jest produkcja kwasu mlekowego podczas fermentacji cukrów oraz związków zapachowo-smakowych. Większość z tych związków powstaje podczas rozkładu aminokwasów pochodzących z białek w procesie transaminacji, oprócz tych, które odpowiadają za smak mleczny i są produkowane podczas fermentacji kwasu cytrynowego. Kwas mlekowy jest czynnikiem konserwującym produkty spożywcze, natomiast substancje zapachowo-smakowe wpływają na właściwości sensoryczne produktu finalnego kształtując smak i aromat. Bakterie mlekowe z gatunku *L. lactis* stanowią przede wszystkim kultury starterowe w produkcji produktów mlecznych takich jak fermentowane napoje mleczne, masło oraz różnego typu sery, a także biorą udział w procesie fermentacji kapusty i ogórków kiszonych.

Substancje zapachowo-smakowe odgrywają bardzo ważną rolę w akceptacji żywności przez konsumentów, dlatego też ich efektywna i tania produkcja odgrywa w dzisiejszych czasach istotną rolę w przemyśle mlecznym. *L. lactis* to najlepiej poznany przedstawiciel bakterii mlekowych odpowiedni do ukierunkowanej modyfikacji metabolizmu w celu poprawienia jakości produktów spożywczych. Celem tej pracy była inżynieria metaboliczna *L. lactis*, która polegała na przekierowaniu fermentacji kwasu cytrynowego w stronę transaminacji aminokwasów w celu produkcji związków zapachowo-smakowych. Dotychczasowa wiedza na temat transaminacji i produkcji substancji aromatyzujących przez *L. lactis* została opisana w rozdziale 1. Częstym czynnikiem limitującym transaminację aminokwasów jest brak wydajnego donora do reakcji czyli α -ketokwasu. Kwas cytrynowy, który jest głównym kwasem organicznym w mleku, ulega rozkładowi w pierwszej kolejności do szczawiooctanu, który jest α-ketokwasem. Zahamowanie aktywności enzymu dekarboksylazy szczawiooctanowej, który to jest odpowiedzialny za rozkład szczawiooctanu do innych intermediantów, okazało się kluczowym czynnikiem w toku moich badań. We wszystkich moich badaniach białko błonowe CitP odpowiedzialne za pobór cytrynianu do komórek bakterii odgrywało znaczącą rolę.

W rozdziale 2 opisane są badania nad fermentacją cytrynianu w laboratoryjnym szczepie dzikim *L. lactis*, które prowadzone były w warunkach obecności lub braku cukru prostego glukozy. Podczas kometabolizmu cytrynianu i glukozy zaobserwowano szybką fermentację cytrynianu do acetoiny. Ten końcowy produkt szlaku metabolicznego nadaje produktom spożywczym smak mleczny. W tych warunkach glukoza ulega rozkładowi do mleczanu w wyniku glikolizy (typ

fermentacji cukrów). Zaobserwowano, iż ogromny wpływ na efektywną produkcję acetoiny odgrywa białko transportujące cytrynian CitP. Szybki import cytrynianu do komórek bakterii odbywa się na zasadzie wymiany między cytrynianem zlokalizowanym na zewnątrz i mleczanem produkowanym wewnątrz komórek. Gdy fermentację cytrynianu przeprowadzono bez udziału glukozy, nie zaobserwowano produkcji acetoiny i transport cytrynianu był wolniejszy. W tych warunkach zmierzono produkcję innych metabolitów takich jak pirogronian, acetomleczan i octan. Pirogronian i acetomleczan to znane intermedianty fermentacji cytrynianu, natomiast octan to produkt końcowy nowego szlaku metabolicznego niescharakteryzowanego wcześniej i opisanego w tym rozdziale. Mimo braku mleczanu produkowanego z glukozy, transport cytrynianu do komórek przez CitP jest wydajny. W tym przypadku import cytrynianu związany jest z eksportem pirogronianu, acetomleczanu i octanu. Następnie przeprowadzono badania powinowactwa substratowego CitP, które wskazały pirogronian, acetomleczan i octan jako nowe substraty, które są transportowane przez CitP. W wyniku badań opisanych w rozdziale 2 scharakteryzowano szlak metaboliczny fermentacji cytrynianu w *L. lactis* w różnych warunkach fermentacji mleka, opisano dwa aktywne szlaki: (1) wcześniej opisany szlak produkujący acetoinę i (2) niezidentyfikowany dotychczas szlak produkujący octan, oraz odkryto nowe substraty CitP, które wspomagają aktywny transport cytrynianu do komórek bakterii. Ponieważ nie zaobserwowano produkcji szczawiooctanu w *L. lactis*, do kolejnych badań opisanych w rozdziale 3 i 5 wybrano mutanta tego samego szczepu z zahamowaną aktywnością dekarboksylazy szczawiooctanowej.

W rozdziale 3 opisano fermentację cytrynianu w szczepie genetycznie zmodyfikowanym, w którym zaobserwowano nadprodukcję α-ketokwasu szczawiooctanu. Wychwyt cytrynianu przez CitP w tym szczepie, w warunkach kometabolizmu z glukozą lub pod jej nieobecność, jest jednakowo wydajny porównując ze szczepem dzikim (opisanym w rozdziale 2). Istotną różnicą okazał się brak produkcji acetoiny podczas kometabolizmu z cukrem prostym. Produktem końcowym, niezależnie od warunków fermentacji cytrynianu, jest octan. Oprócz tego zmierzono, iż co najmniej 80 % szczwiooctanu jest wydalane z komórek bakterii, a następnie związek ten jest pobierany do wewnątrz komórek i powoli rozkładany do octanu w wyniku działania innej nieznanej dekarboksylazy o niskim powinowactwie do szczawiooctanu. Jednoczesny import cytrynianu i eksport szczawiooctanu zasugerował, że proces ten katalizowany jest przez CitP. Ponieważ białko transportujące szczawiooctan nie zostało wcześniej scharakteryzowane w *L. lactis*, przeprowadzono dalsze badania powinowactwa substratowego CitP, które potwierdziły, że α-ketokwas jest nowym substratem. W wyniku mutacji dekarboksylazy szczawiooctanowej w *L. lactis*, szczawiooctan produkowany z cytrynianu jest szybko akumulowany w cytoplazmie komórek. Duże ilości tego związku są toksyczne dla *L. lactis*, dlatego też, szczawiooctan jest wydalany przez CitP na zasadzie wymiany z cytrynianem na skutek odpowiedzi stresowej. Następnie, związek ten jest pobierany przez CitP do komórek i powoli rozkładany do octanu. W toku badań opisanych w rozdziale 3 zanalizowano metabolizm kwasu cytrynowego w szczepie genetycznie zmodyfikowanym, zaobserwowano produkcję α-ketokwasu szczawiooctanu, który

może być przekierowany do produkcji związków zapachowo-smakowych jak założono w powyższej pracy doktorskiej, co dalej jest opisane w rozdziale 5 oraz scharakteryzowano szczawiooctan jako nowy substrat CitP.

W rozdziale 2 i 3 zademonstrowano, że białko błonowe CitP może transportować różne substraty takie jak cytrynian, mleczan (wcześniej opisane w literaturze) oraz pirogronian, acetomleczan, octan czy szczawiooctan (badania opisane w tej pracy). Ponieważ zdolność transportowania wielu związków o różnej strukturze chemicznej jest unikatowa, w rozdziale 4 przeprowadzono szczegółową analizę powinowactwa substratowego i opisano mechanizm transportu CitP. W tych badaniach zademonstrowano 22 nowe substraty tego białka. Większość z badanych związków odgrywa istotną rolę w przemyśle spożywczym. CitP odpowiada za eksport substancji zapachowo -smakowych z komórek bakterii *L. lactis*, które powstają na skutek rozkładu aminokwasów w procesie transaminacji (α-ketokwasy pochodne od aminokwasów) lub ich dalszego rozkładu (α-hydroksykwasy pochodne od α-ketokwasów). Związki te wpływają korzystnie na smak i aromat produktów mlecznych. Inne substraty CitP to kwas benzoesowy, mrówkowy, jabłkowy, fumarowy, cytrynowy, adypinowy i bursztynowy, które stosowane są jako środki konserwujące żywność, regulatory kwasowości, wzmacniacze smaku i przeciwutleniacze w produktach spożywczych. Z badań przeprowadzonych w rozdziale 4 wynika, iż białko błonowe CitP prezentuje szerokie powinowactwo substratowe dla związków niezwykle ważnych w przemysłowej produkcji żywności.

Główną tezę rozprawy doktorskiej czyli przekierowanie metabolizmu kwasu cytrynowego w stronę transaminacji aminokwasów do produkcji związków zapachowo-smakowych zademonstrowano i opisano w rozdziale 5. Badania w tym rozdziale przeprowadzono w szczepie laboratoryjnym genetycznie zmodyfikowanym, który wykazał nadprodukcję α-ketokwasu szczawiooctanu produkowanego z cytrynianu, co scharakteryzowano w rozdziale 3. Kwas cytrynowy i aminokwasy, które powstają z rozkładu białek, są obecne w mleku. Najbardziej wartościowe substancje zapachowo-smakowe pochodzą z rozkładu aminokwasów takich jak izoleucyna, leucyna, walina, fenyloalanina, tryptofan, tyrozyna i metionina, dlatego też badania prowadzono w obecności tych aminokwasów. Transaminacja to pierwsza reakcja w łańcuchu produkującym aromaty, która katalizowana jest przez enzymy transaminazy. W toku tej reakcji α-ketokwas szczawiooctan jest przekształcany w aminokwas kwas asparaginowy, natomiast aminokwasy (wyżej wymienione) są przekształcane w odpowiednie im α-ketokwasy. Następnie α-ketokwasy pochodne od aminokwasów są przekształcane w inne związki aromatyzujące takie jak α-hydroksykwasy, aldehydy, estry i pochodne kwasy karboksylowe. Podczas kometablizmu kwasu cytrynowego i aminokwasów w mutancie *L. lactis* zaoberwowano wydajną transaminację szczawiooctanu i produkcję α-ketokwasów pochodnych od wszystkich wykorzystanych aminokwasów. Dodatkowo, α-ketokwasy pochodne od fenyloalaniny i metioniny uległy rozkładowi do α-hydroksykwasów lub aldehydów. Eksport α-ketokwasów i α-hydroksykwasów odbywa się na zasadzie wymiany z cytrynianem lub szczawiooctanem, a reakcja ta katalizowana jest przez białko błonowe CitP, co zademonstrowano również w rozdziale 4. W rozdziale 5 przedstawiono,

że wydajna produkcja substancji zapachowo-smakowych podczas kometabolizmu cytrynianu i aminokwasów jest możliwa w *L. lactis*, a także ogrywa pozytywną rolę fizjologiczną w uwolnieniu toksycznego stresu wywołanego przez akumulację szczawiooctanu w cytoplazmie. Wszystkie związki produkowane w toku tych badań w procesie transaminacji odpowiadają za odbiór sensoryczny produktów spożywczych. Na przykład jednym z produktów tej reakcji jest aldehyd metioniny, który odpowiada za aromat pieczonych ziemniaków, DMDS produkowany z metioniny, który odpowiada za aromat cebulowy, siarkowy czy aldehyd benzoesowy produkowany z fenyloalaniny, który nadaje smak gorzki, migdałowy i aromat słodkich czereśni. Zaobserwowano, że transaminazy *L. lactis* przeprowadzające proces transaminacji mają powinowactwo do szczawiooctanu. Pokazano również, że białko transportujące CitP ogrywa bardzo ważną rolę podczas produkcji substancji zapachowo-smakowych przy udziale cytrynianu (szczawiooctanu), ponieważ odpowiada za import prekursorów reakcji (cytrynian, szczawiooctan) i eksport aromatów (α-ketokwasy, α-hydroksykwasy).

W rozdziale 6 przedstawiono badania procesu transaminacji aminokwasów z udziałem innego α-ketokwasu – kwasu α-ketoglutarowego, który jest scharakteryzowany jako najlepszy i najbardziej wydajny prekursor związków zapachowo-smakowych, co potwierdzono badaniami *in vitro*. Ponieważ białko transportujące ten prekursor do komórek bakterii mlekowych był dotąd nieznany, nie można było przeprowadzać efektywnej produkcji substancji zapachowo-smakowych podczas procesu wytwarzania produktów fermentowanych, głównie serów, z udziałem tego prekursora. Podczas transaminacji tego α-ketokwasu w szczepie dzikim *L. lactis*, w którym znajduje się białko błonowe CitP, zaobserwowano znaczną produkcję α-ketokwasów pochodnych od aminokwasów takich jak izoleucyna, leucyna, walina, fenyloalanina, tryptofan, tyrozyna i metionina. Gdy transaminację kwasu α-ketoglutarowego i aminokwasów przeprowadzono w warunkach obecności cukru prostego glukozy, zmierzono bardziej wydajną produkcję związków zapachowo-smakowych. Dodatkowo w tych warunkach zauważono produkcję α-hydroksykwasów z α-ketokwasów pochodnych od aminokwasów. Badania przeprowadzone w pochodnym szczepie *L. lactis* bez białka błonowego CitP zasugerowały, że kwas α-ketoglutarowy może być kolejnym substratem CitP. Analiza powinowactwa substratowego CitP dla tego kwasu potwierdziła tą hipotezę. Podsumowując, w rozdziale 6 scharakteryzowano białko transportujące kwas α-ketoglutarowy oraz efektywną produkcję substancji zapachowo-smakowych podczas transaminacji tego prekursora.

Badania opisane w rozdziałach 2-6 są opublikowane w czasopismach naukowych. W rozdziale 7 podsumowano znaczenie procesu transaminacji szczawiooctanu i pirogronianu produkowanego podczas fermentacji cytrynianu w produkcji związków zapachowo-smakowych w przemyśle mlecznym oraz opisano transaminazy zaangażowane w ten proces.

List of publication:

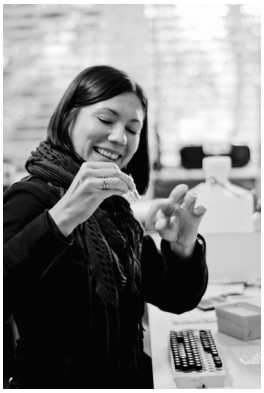
- **Pudlik A. M.** and J. S. Lolkema. 2012. Uptake of α-ketoglutarate by the citrate transporter CitP drives transamination in *Lactococcus lactis*. Submitted to *Appl. Environ. Microbiol*.
- **Pudlik A. M.** and J. S. Lolkema. 2012. Rerouting of citrate metabolism in *Lactococcus lactis*: citrate driven transamination. Accepted to *Appl. Environ. Microbiol*., published ahead of print on July 13th.
- **Pudlik A. M.** and J. S. Lolkema. 2012. Substrate specificity of the citrate transporter CitP of *Lactococcus lactis*. *J. Bacteriol.* 194(14):3627-35.
- **Pudlik A. M.** and J. S. Lolkema. 2011. Mechanism of citrate metabolism by an oxaloacetate decarboxylase-deficient mutant of *Lactococcus lactis IL1403*. *J. Bacteriol.* 193(16):4049-56.
- **Pudlik A. M.** and J. S. Lolkema. 2011. Citrate uptake in exchange with intermediates in the citrate metabolic pathway in *Lactococcus lactis* IL1403. *J. Bacteriol.* 193(3):706-14.
- **Pudlik A. M.**, K. Hupert-Kocurek, and S. Łabużek. 2008. Wektory ekspresyjne w bakteryjnych systemach nadprodukcji białek. Laboratorium, 6:32-40.

Patent application:

Pudlik A. M., J. S. Lolkema, M. Kleerebezem, L. Sijtsma. 2012. Process for the preparation of a food product. Patent no. 12163465.3-1221.

Nomination for the TIFN Publication Prize 2012 and the TIFN Poster Prize 2012.

About the author



Agata Maria Pudlik was born in Gliwice, Poland, on the 2nd of August 1984. She attended the class of mathematics and computer sciences in High School No. 4 in Gliwice, graduating in 2003. Simultaneously, since she was 7 years old, she attended the Music School in Gliwice. The first six years she played violin and piano, the next six years she played percussion instruments in the class of M.A. W. Morcinczyk. Later on, she decided to study two disciplins: Percussion Instruments at the Academy of Music in Katowice under the supervision of Dr. K. Jaguszewski and Prof. Dr. S. Proksa and Biotechnology at the University of Silesia in Katowice. In 2005 she chosen to focus on science and she started one year training at the Molecular Biology Department from the Comprehensive Cancer Centre in Gliwice ahead of Prof. Dr. S. Szala. She obtained her degree of Bachelor of Science in Biotechnology in 2006. During this study she performed her thesis “The bacterial recombinant expression vectors” at the Department of Biochemistry under the supervision of Dr. K. Hupert-Kocurek and Prof. Dr. S. Łabużek. She continued with the master study in Biotechnology at the University of Silesia in Katowice. At that time, she performed one year research internship at the University of Bayreuth in Germany to study “Molecular genetic analysis of the extracytoplasmic domain of the *Bacillus subtilis* anti-sigma factor RsiW” under the supervision of Dr. T. Wiegert and Prof. Dr. W. Schumann. In 2007, during her master study in Poland, she was one of the initiator of the Biotechnology Student Association under the supervision of Prof. Dr. Z. Piotrowska-Seget at the Silesian University to study “Bacterial heavy metal resistance systems”. She completed the study with master courses at the Department of Genetics in Bayreuth and the Department of Biochemistry in Katowice. She obtained her Master’s degree in July 2008.

Thereafter, from August 2008 until July 2012, she worked on her PhD studies at the Department of Molecular Microbiology from University of Groningen under the supervision of Dr. J. S. Lolkema founded by the Top Institute Food and Nutrition. The research performed during this period is described in this thesis.

Currently, the author is employed as a technologist in the Research and Implementation Center from Mokate Sp. z o.o. in Żory, Poland.

Courses:

Academic Teaching Skills & Course Design	2012
Mass Spectrometry	2011
Microorganisms in Food Production and Preservation	2011
Single Molecule & Single Cell Analysis	2010
Advanced Techniques in Bacterial Genome Research	2010
Transport Processes in Industrial Microorganisms (B-Basic Masterclass)	2010
Publishing in English	2010
Presentation Skills	2010
Current Membrane Protein Research (EMBN Workshop)	2009
Radiation Protection Course (level 5B)	2008

Meetings:

The 3rd TIFN Annual Conference, Arnhem, The Netherlands (poster)	2012
LAB10 meeting, Egmond, The Netherlands (poster)	2011
Gordon Research Conference on Mechanisms of Membrane Transport, Biddeford, USA (poster)	2011
The use of microorganisms and their metabolites in food production and preservation, Kiry, Poland (oral presentation)	2011
NWO Scientific Meeting: Chemistry related to Biological & Medical sciences, Veldhoven, The Netherlands (poster)	2010
ESF-Bielefeld-CeBiTec Conference on Microbes and Industrial Biotechnology, Bielefeld, Germany (poster)	2010
18th Annual GBB Symposium, Groningen, The Netherlands (poster)	2010
B-basic Masterclass: Transport Processes in Industrial Microorganisms, Groningen, The Netherlands (poster, oral presentation)	2010
TIFN MidTeRev, Wageningen, The Netherlands (oral presentation)	2010
NWO Scientific Meeting: Chemistry related to Biological & Medical sciences, Veldhoven, The Netherlands (oral presentation)	2009
17th Annual GBB Symposium, Groningen, The Netherlands (poster)	2009
EMBN Workshop: Current Membrane Protein Research, Groningen, The Netherlands (poster)	2009
NWO Scientific Meeting: Chemistry related to Biological & Medical sciences, Veldhoven, The Netherlands (poster)	2008
16th Annual GBB Symposium, Groningen, The Netherlands	2008

Optional activities:

Assistance during the LAB10 symposium	2010
TIFN project meetings	2008-2012
TIFN WE-days (oral presentations)	2008-2011
MolMic lab meetings	2008-2012

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